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SOME EFFECTS OF $HgCl_2$ ON FERTILIZED AND UNFERTILIZED EGGS OF *ARBACIA PUNCTULATA*

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In connection with a study of the fertilization reaction in *Arbacia punctulata,* the writer reported, in a previous number of this journal (Hoadley, 1923), some observations on the relationship between the concentration of salts of the heavy metals and frequency of cleavage in the egg of that form. In the course of these experiments it was found that mercury had certain effects upon cleavage which appeared to be indicative, though sufficient information was not available at that time to warrant any discussion of the phenomena involved. During the past summer, a study of these effects has been made. The results of this investigation will be presented below.

In a series of the heavy metals, $HgCl_2$ stands apart from the other chlorides tested in respect to its action on cleavage and membrane elevation. Thus it is found that there is no concentration of the mercuric salt which inhibits membrane elevation while permitting cell division. Quite on the contrary, the presence of the mercuric ion appears at certain concentrations, to favor membrane elevation, resulting in much wider membranes than is the case in sea-water alone. Cleavage, however, is completely inhibited in solutions of relatively low salt content. When a series of concentrations is tested, it is found that the inhibition of cleavage is associated with certain changes in the egg which are peculiar to mercuric chloride, not being identified with the action of any of the other salts studied. The definite nature of these changes is most evident in solutions of $HgCl_2$ between M/30,000 and M/50,000. These concentrations are from three to six times as great as those required to suppress cleavage completely in eggs exposed

[1] This investigation was aided by grant from the Milton Fund.

to the solution from the time of complete membrane elevation on. I would like at this point to describe very briefly the general picture presented by eggs under such conditions.

Eggs of *Arbacia*, transferred to a solution of $HgCl_2$ in sea-water (M/40,000) five minutes after insemination in sea-water, show well-elevated membranes. The nuclear region is just becoming visible in the living egg in the form of a bright spot. The pigment is composed of very small granules which are evenly distributed throughout the cortical portion. After approximately thirty minutes the entire picture is changed, being very different from that found in the control eggs. The pigment is clumped at one side of the egg, where it is bulging outward as though the egg were cleaving into two parts, a larger one containing little or no pigment and a smaller containing all of the pigment which appeared in the cortical portion at the time of transfer. After 10 minutes in this condition, the pigment again becomes distributed throughout the entire cytoplasmic portion of the egg which proceeds to cytolyze. The somewhat clumped pigment loses its red color and fades materially early in the process. The pseudo-cleavage and the behavior of the pigment prior to and after the pseudo-cleavage and the accompanying cytoplasmic phenomena involved are of especial interest.

Experiments

In the present study, as in that cited above, the egg of *Arbacia* was used. Solutions of $HgCl_2$ were made in sea-water from an M/10 stock solution in distilled water. The sea-water used in the dilutions had been standing in the room for a considerable period of time to bring it to the temperature of the air. Because of previous experience, attention was focused on the behavior of fertilized eggs in $HgCl_2$ concentrations between M/30,000 and M/50,000.

The description of the experimental results will be made according to increase in the concentration of mercuric salt in the solution employed. It should be borne in mind that the weakest, M/50,000, is a concentration which is three times as great as that necessary to completely suppress cleavage in fertilized eggs placed in it directly after complete membrane elevation. The experiments were performed in the following way.

Sperm and eggs were obtained from fresh *Arbacia* after washing the individuals and all instruments in running fresh water to kill adhering gametes, and then in sea-water to guard against any effects of hypotonicity. The eggs were obtained by straining broken ovaries through cheese-cloth. They were subsequently washed in three changes of sea-water to carry off body fluid and small pieces of the gonads.

The eggs were then examined to be sure none had membranes elevated and to ascertain the condition of the gametes. No batches of eggs were used which showed one per cent in germinal vesicle stages. With due precaution no eggs will be found with membranes elevated as a result of sperm infection or hypotonicity. The eggs were inseminated 20 minutes (ca.) after the removal of the gametes from the ovary. A sperm suspension, made by adding one drop of dry sperm to 25 cc. of sea-water having been prepared, 7 drops of this were added to 50 cc. of sea-water into which the eggs from a single female had been placed. One control of uninseminated eggs in sea-water, and one in the $HgCl_2$ solution were reserved for subsequent examination. Observations were then made to determine the fertility of the eggs. No batch with less than 98 per cent of the membranes elevated was employed. The eggs were then allowed to stand in sea-water until 5 minutes after insemination, when the transfer to the $HgCl_2$ was made. When insemination is carried out as stated above it will be described in the following way: 7(1:25):50.

The transfer of eggs to the $HgCl_2$ solutions and the retransfer to sea-water were accomplished as follows. Five minutes after insemination, 5 drops of the inseminated eggs were transferred to 10 cc. of $HgCl_2$ in sea-water and the eggs evenly arranged in the dish. This last precaution is very necessary in order to assure comparable results, for crowding affects not only the respiratory activities of the eggs, but also the concentration of the Hg^{++} ion in the immediate neighborhood, the initial amount being very small and the eggs containing substances which, as we shall see, are mercury-avid. The retransfers to sea-water were made at 3-minute intervals. One drop of eggs from the mercury solution was placed in 7.5 cc. of sea-water. All dishes were kept covered to prevent evaporation, and were also kept out of direct sunlight to prevent much change in temperature. The results obtained at any one concentration are so nearly the same that but one series of experiments will be described under each.

The information obtained in the present experiments may be presented in two ways, either of which would yield comparable data. We may consider each of the molecular concentrations of the salts, varying the period of exposure, or we may compare similar periods of exposure, varying the concentration of the solutions employed. Both methods of procedure have been employed. In general, however, the first method leads to greater simplicity, and will therefore be used in the description. A word should be said about the differences in response in batches of eggs from different females. This is, of course, to be expected, and might be satisfactorily explained could the relative

amount of pigment be computed as well as allowance made for differences in the states of the gametes when used.

M/50,0000 $HgCl_2$.—This concentration was made up in sea-water from a M/10 $HgCl_2$ solution in distilled water. The temperature of the solution was 23° C. Eggs and sperm were prepared as described above and insemination made 7(1:25):50. Uninseminated controls in sea-water showed zero per cent membranes after 15 minutes. Inseminated controls showed 100 per cent membrane elevation 5 minutes after insemination. Uninseminated eggs in the $HgCl_2$ showed 0.5 per cent membrane elevation after 20 minutes' exposure. Five minutes after the sperm were added to the eggs, 5 drops of eggs were put into the M/50,000 $HgCl_2$ solution. This constituted the stock from which transfers were made to sea-water at 3-minute intervals up to 35 minutes. We shall now consider the effect of the solution on the eggs remaining in it, the changes on the return of the eggs to sea-water, the rate of cleavage in the eggs after each exposure, and the extent of their development.

Mercuric chloride of this strength has a very definite effect on the rate with which nuclear changes occur in the inseminated eggs. It

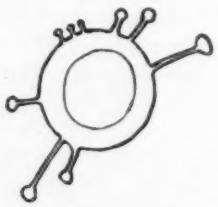

FIG. 1. Egg in the germinal vesicle stage showing the long processes expelled when egg is placed in the $HgCl_2$ solution.[2]

is not as marked as in more concentrated solutions, but, over an extended period, e.g., 25 to 30 minutes, it is evident that the whole succession of nuclear phases is retarded. In this connection it should be mentioned that the effects are immediately made manifest in eggs in germinal vesicle stages (see Fig. 1 and note) and in eggs approaching the period of division. Save for the slight retardation, there is no

[2] It is true that when *Arbacia* eggs are placed in sea-water a certain number of the eggs in the germinal vesicle stage will throw out processes similar in every way to those pictured here, but it is not true of all of the eggs in this condition. In the mercuric chloride solution it is the case with all of them. Whether this is a direct action of the solution on the more superficial regions of the egg or whether this is due to the indirect action of the salt on the membrane and subsequently the balance between the physical states of the cytoplasm and the medium is not clear.

evidence of any alteration of the eggs in the toxic solution until from 27 to 29 minutes, when 10 per cent of the eggs showed localization of pigment (cf. Fig. 2). With the increase in exposure time, the number of eggs showing the direct effect of the solution mounts thus, 32 minutes—30 per cent, and 35 minutes—40 per cent. With the localization of the pigment, cytolysis may be seen to be taking place around the clumped granules. The rest of the cortical portion of the egg remains intact. After long exposure, the intact eggs swell greatly, the contents being visibly coagulated and quite free of pigment. Fragmentation may be observed in some of the eggs in which pigment

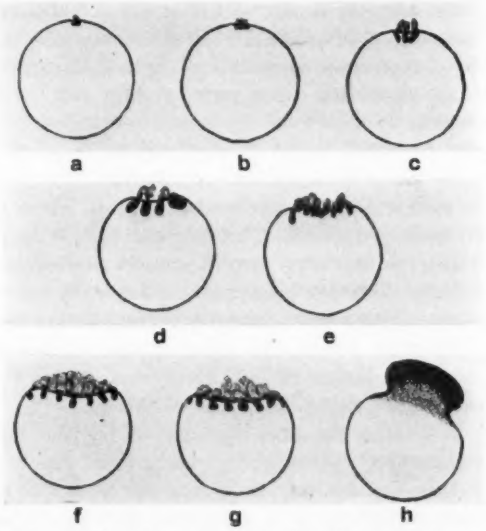

FIG. 2. Series showing the progressive effect that a series of exposure times has upon the localization of the pigment and the accompanying local cytolysis. In *h* may be seen the general picture presented by an egg undergoing "pseudo-cleavage." For explanation see text.

localization takes place. This is much more common in some of the experiments to be described below. No true cleavage ever is seen in eggs which remain in the $HgCl_2$ solution.

The effect of solutions of mercuric salt is not immediately apparent on the eggs. This is particularly noticeable in cases where the eggs are returned to sea-water after short exposures. It may be seen in the behavior of the pigment and subsequently in the rate and percentage of cleavage, as well as in the viability of the cleaving stages. It will

be recalled that in the $HgCl_2$ solution, noticeable clumping of pigment appears at from 28 to 29 minutes. In the transferred eggs, however, some 3 per cent show a great deal of pigment localization with pseudo-cleavage at an age of 45 minutes, even when the transfer to sea-water has taken place after 18 minutes' exposure. Thirteen minutes later, these same eggs showed 55 per cent localized pigment. In eggs which were transferred after 15 minutes' exposure, while no pseudo-cleavage was found, 48 per cent showed localized pigment with cytolysis after 57 minutes. In eggs which have been exposed to the $HgCl_2$ solution for longer periods of time, the percentage showing pseudo-cleavage and localized pigment increases directly with the length of exposure. This last involves not only the extent of the action in individual eggs, but also the number of eggs affected. Generally speaking however, the extent of the phenomenon in individual eggs is directly comparable after similar exposure times. This varies slightly with different batches of eggs employed.

The extent to which pigment clumping takes place indicates a mechanism responding in a purely quantitative fashion. In Fig. 2 may be seen a series of effects produced in eggs on return to sea-water after successively longer periods of exposure to M/50,000 $HgCl_2$. The clumping itself is but the initial step in a series of changes which will be described here. Before the actual accumulation of the pigment takes place, there is little evidence of migration within the cytoplasm of the egg. In other words, the pigment which takes part in this action does not form larger masses which move through the cortex, but the massing occurs only at the point of final grouping. The result is that there is no apparent local depletion of pigment, but rather, a general depletion which takes place evenly over the entire cortex. After a short exposure this may be similar to that shown in Fig. 2, *a*, while after longer exposure it is more extensive, as shown in subsequent figures. As might be expected, with the increase in the amount of the pigment activated, the area of the cortical region involved is increased. The clumped pigment, however, is evidently destined to expulsion by the cytoplasm. As it reaches the surface in the particular area shown, it breaks through the membrane as globules. These immediately swell and undergo changes which are customarily described in eggs as cytolytic. The area of "cytolysis" is directly referable to that of pigment localization, the remainder of the cortex remaining intact. In extreme cases where exposure is long (e.g. 30 minutes), all of the visible pigment may be removed from the cytoplasm, and instead of a "local cytolysis" of the area involved, this area may be budded off as seen in Fig. 2, *h*. If this budding is extensive it may

result in what I have called a pseudo-cleavage; if it is not as extensive, the budding may not be complete, the pigment may again enter the pigment-free portion, and cytolysis follow around each of the pigment masses. The most interesting observations associated with this action are, that pigment is affected evenly throughout the cortex; that it is eventually localized at one region of the cortex; and that it is eliminated at this point, the elimination being accompanied by cytolytic changes. It would be of great interest to determine at just what point in the cortex the accumulation of the pigment takes place. Is it in any constant relation to the polar axis of the egg? This is of even greater importance after a consideration of certain facts concerning the subsequent cleavage. There is, however, no definite evidence available on this point. An hypothetical determination might very easily lead to great error. This will be mentioned again in connection with cleavage.

Not all of the eggs which show the effects of mercury action fail to cleave. It has been mentioned above that no cleavage takes place in $HgCl_2$ solutions employed, so that we may confine our consideration to those eggs which have been returned to sea-water. Two sorts of data, both dependent on cell division, may be obtained from the experiment. The solution reduces the total number of eggs cleaving, and, in addition, it increases the length of the period between fertilization and cleavage. In the series already considered above, the percentage

TABLE I

Effect of M/50,000 $HgCl_2$ on the time of the appearance of the first cleavage in eggs exposed to the solution for various periods of time. Time in minutes after insemination.

Exposure *minutes*	First egg observed in first cleavage	
0	42	minutes
3	48.5	"
6	50.	"
9	53.	"
12	55.	"
15	68.	"
18	71.	"
21	74.	" (attempted)

of cleavage drops constantly from 100 per cent after 9 minutes' exposure, to zero per cent after 24 minutes' exposure. There were only a few eggs which showed any cleavage after 21 minutes' exposure, and in those cases the retardation was very great, the time not being recorded because of the fact that all eggs were thought to be dead. The length of period required for the cleavage shows much variation, but the figures to be seen in Table I, for example, represent the time of

appearance of the first cleaving egg seen in each group of one batch. The retardation is marked. The interpretation of conditions within any one dish of eggs is further complicated by the fact that not all of the eggs which show the localized cytolysis around the pigment, cytolyze completely. In many cases in which cytolysis does not become more extensive than is shown in Fig. 2, *a*, *b*, *c*, and even *d*, the elimination of pigment is not fatal to the egg, which subsequently divides. This recovery, if it may be called such, appears to be more complete, and sooner complete, the less pigment is involved in the cytolysis. Thus, in one case, after an 84 per cent localized pigment cytolysis of the type shown in Fig. 2 *b*, 94 per cent of the eggs cleaved. Such behavior suggests the question as to possible relation between point of cytolysis and plane of cleavage, a point which I shall discuss here for all of the material considered.

There is a definite relation between the cleavage planes and the point of pigment accumulation and cytolysis. Whether this indicates an orientation of this point to polar orientation of the egg, or an effect of the localization of the pigment clumping on the orientation of the cleavage spindle, is not indicated in any of the experiments. In either case, the subsequent cleavages occur as shown in Fig. 3, *a*, *b*, and *c*.

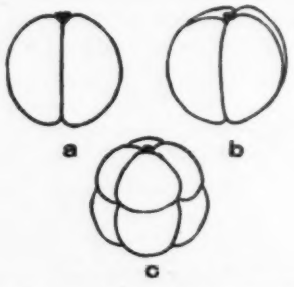

FIG. 3. Sketches to show the relationship between the point of pigment extrusion and the first, second and third cleavage planes. For explanation see text.

The relation of the extruded pigment is plainly visible because it remains attached to the blastomeres for a considerable length of time. All evidence obtained seems to point to the probability that this pigment is of relatively little significance as far as the early development of the germ is concerned. Cleavages follow as in typical development and motile larvæ are formed. A more complete discussion of this point will be found below.

The number of motile forms produced after exposure of eggs to

the mercuric chloride also expresses the toxicity and inhibition already observed above. Table II is a record of conditions in the cultures 18 hours after insemination. Any exposure up to 9 minutes may be seen, on reference to the table, to have relatively little effect on future development. With the advance to 12 minutes' exposure, however, the number of viable individuals has dropped to 60 per cent, and these show marked retardation. After 15 minutes' exposure the retardation

TABLE II

Effect of M/50,000 $HgCl_2$ on the rate of development of larvæ after exposure of the eggs for various times as indicated. The examination of the culture was made eighteen hours after insemination of the eggs.

Exposure *minutes*	Motile *per cent*	Extent of development
0	100	Young larvæ
3	100	" "
6	100	" "
9	90	" "
12	60	Very late gastrulæ
15	40	Early gastrulæ
18	30	Blastulæ
21	1	Early blastulæ
24	0	

is even greater, as is also the case after 18 minutes. Viability also decreases. The possible relationship between the retardation and the action of the pigment will be considered below.

M/45,000 $HgCl_2$.—As in the experiment just described, this concentration of $HgCl_2$ was made up in sea-water from an M/10 stock solution in distilled water. The temperature of the sea-water used in the dilution was 22° C. Eggs and sperm were prepared with the customary precautions. After controls of the uninseminated eggs had been set aside, the balance of the gametes were placed in 50 cc. of sea-water to which sperm suspension was added according to the formula 7(1:25):50. The eggs were all mature and showed 100 per cent membrane elevation 5 minutes after the addition of the sperm. The uninseminated eggs in sea-water showed zero per cent membrane 20 minutes later. No membranes were observed on uninseminated eggs in $HgCl_2$ 20 minutes after transfer. Five minutes after insemination, 5 drops of the eggs were added to 10 cc. of the M/45,000 $HgCl_2$ solution and these were evenly distributed on the bottom of the container. From this lot, samples were returned to sea-water at 3-minute intervals until 10 lots were available for study. The results of the observations follow.

The mercuric chloride in this concentration has a far greater in-

hibiting effect than in that dealt with in the previous section. This is noted first on observation of the nuclear changes in the egg. After about six minutes in the solution, the eggs show the monasters characteristic of the earliest phases and with this the progression of stages stops. If the eggs are observed at intervals one finds that gradually changes occur which concern the distribution of the pigment in the cortical cytoplasm, but these do not involve further changes in the nucleus. Those eggs in which the pigment does not clump gradually swell and eventually show a coagulated cytoplasm within a much bloated membrane. In by far the greater number of the eggs the swelling is preceded by a pigment clumping which is much more extensive in this case than in that previously described. After 24 minutes in the solution, about eight per cent of the eggs show a localization of the pigment which is not always confined to one spot as was the case in the weaker solution, but may be located at two or three regions, immediately below

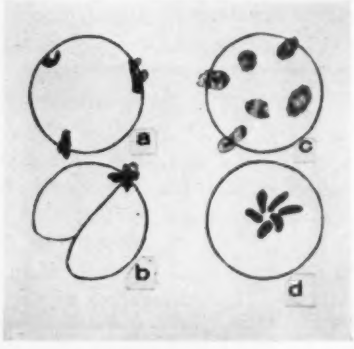

FIG. 4. Sketches to show more extensive action of the solution on the eggs. In *a* and *c* may be seen various degrees of multiple pigment foci. In *b* is an egg with purely cytoplasmic cleavage, a process resembling fragmentation. In *d* is a "polar" view of an egg to show the general picture presented by the clumped pigment. For explanation see text.

the egg membrane (Fig. 4, *a*). This action is associated in a very small number of the eggs with a pseudo-cleavage of the type described above, or with a constriction of the pigment-free portion of the egg such as may be seen in Fig. 4, *b*. The extent of the occurrence of such forms increases with the length of the exposure as does also the frequency of the eggs with multiple pigment centers, so that after some 40 minutes, when all of the eggs are swelling and none of them appear at all normal, some eggs may be found with as many as six of these

centers arranged as in Fig. 4, *c*. These forms are always much rarer than those of the pseudo-cleavage type.

It is not as easy to follow the course of the pigment clumping in eggs in this concentration as it was in the previous one. This is true for two reasons. A difference of 3 minutes in the exposure time in the higher concentration is equal in its effects to that of a 12-minute interval in the lower concentration. Moreover, the pigment changes occur very rapidly after the eggs are returned to the sea-water, and the duration of the intermediate stages is shortened. Hence their determination is rendered more difficult. Associated with this more rapid reaction within the egg after its return to sea-water, we find an increase in the number of eggs in which the clumping of the pigment is complete. Thus there is a rise to 20 per cent of pseudo-cleavage in eggs that have been exposed to the mercuric solution for 24 minutes. This complete isolation of the pigment appears for the first time in eggs which have had a 15-minute exposure to the solution. Some effect on the pigment is found even in the 6-minute eggs. It is not extensive, resembling in most of its details the picture presented by the eggs in Fig. 2, *a*. Of these eggs, 40 per cent were so affected while 98 per cent of them cleaved. In the 9-minute eggs the condition is much more advanced. Pigment clumping is accompanied by local cytolysis as in the previous cases. The situation is very much more general here, being found in 96 per cent of the eggs. There is a 100 per cent local cytolysis in the 12-minute eggs which is far more extensive in its nature than anything described in the discussion of the first series. A rather typical egg may be seen in Fig. 4, *d*. Further exposure tends to produce even more extensive clumping of the pigment, with pseudo-cleavage, and with the multiple pigment centers. These last are found in eggs which appear for the most part to have a coagulated cytoplasm. It looks very much as though pigment localization having been initiated, coagulation had set in so that migration was stopped, leading to pigment clumping around a number of centers. Local cytolysis occurs around each of the pigment masses and swelling follows. None of these eggs ever show any further developmental changes.

The effect of the $HgCl_2$ on cleavage is much more extensive in these concentrations than in the weaker ones. Immediately following the retransfer of the eggs to sea-water, there is a rapid change in the nuclear components, so that bar and streak stages are formed. This takes place only in those eggs which have been in the solution for 15 minutes or less, though an occasional egg is found which will proceed after 18 minutes in the toxic solution. There is a progressive retardation as the length of exposure increases. Thus, the 3-minute group

cleaved 9 minutes after the controls, while the 15-minute group was retarded 75 minutes. In the 18-minute group, only an occasional egg cleaved, and then 135 minutes after division in the controls. The effect may most readily be seen in the percentage of cleaving eggs. This drops from 98 per cent after 3 and 6 minutes' exposure, to 90 per cent after 9 minutes', 72 per cent after 12 minutes', 18 per cent after 15 minutes', and less than one per cent after 18 minutes'. The percentage of cleavage is, therefore, inversely proportional to the length of the exposure to the mercuric solution. Inasmuch as there is a 40 per cent local cytolysis in the 6-minute eggs, 96 per cent in the 9-minute eggs, and 100 per cent in the 12-minute eggs, it is evident that many of those which have shown local cytolysis must cleave. This has been observed here as in the cases described above. The attached extruded cytolysing pigment can be traced through at least the 16 and 32-cell stages, as was the case above (cf. Fig. 3). Additional information on viability is available for these eggs, however, for observations were made periodically up to the time of pluteus formation.

Motile larvæ were produced by cultures of eggs removed from the $HgCl_2$ solution after 3, 6, 9, and 12 minutes' exposure. Of these, only the first three continued in their development to the formation of plutei. Forty-eight hours after insemination in the first two cultures, *i.e.*, in those of 3 and 6 minutes' exposure to the solution, the plutei produced appeared typical in every way. In the 9-minute culture, on the other hand, the few plutei formed were much retarded. The arms were short and the body heavy. A relatively small number of the larvæ continued their development to this point. It will be recalled in this connection that before cleavage, 96 per cent of these eggs showed localized pigment with an associated cytolysis which was quite extensive. In the 6-minute group, the localized cytolysis and pigment clumping had affected 40 per cent of the eggs. While a definite statement cannot be made about the 9-minute culture, it was perfectly evident that over 60 per cent of the 6-minute eggs had formed plutei. The experiment shows that typical plutei can be formed from the eggs which have undergone the pigment localization and localized cytolysis produced by short exposure to M/45,000 $HgCl_2$ solutions.

M/40,000 $HgCl_2$.—The temperature of this concentration, which was made up in sea-water from the M/10 stock solution of the salt was 21.3° C. Eggs and sperm were prepared as before and insemination carried out according to the same formula, 7(1:25):50. The usual controls of uninseminated eggs were set aside. That in sea-water showed no membranes elevated after a period of 30 minutes. In $HgCl_2$ solution, examination of the material showed 8 eggs with wide mem-

branes (less than one per cent) after 15 minutes' exposure. Inseminated controls showed 100 per cent membrane elevation after 5 minutes and 100 per cent cleavage at 45 minutes. Five minutes after insemination, 10 drops of eggs were added to 10 cc. of the $HgCl_2$ solution. These were returned to sea-water at 3-minute intervals until 10 transfers had been made. The results of the experiment are similar in many ways to those already described above, save that the modifications in the eggs appear earlier. After longer exposures, there are some differences which will be discussed below.

The transfer of the eggs to the $HgCl_2$ solution is immediately followed by an increase in the width of the space formed by the elevation of the membrane. Results of a similar nature were obtained in previous experiments already referred to in the introduction. The increase is much greater than any noted at weaker concentrations. In the meantime, the nuclear elements appear as monasters. This is as far as the nuclear changes go in the solution, no bar or streak being formed. The eggs remain in this condition until the total exposure amounts to approximately 24 minutes, when the pigment begins to show the clumped effect typical of longer exposures in less concentrated solutions. Subsequently the eggs appear to be damaged rapidly, swelling and coagulation being evident in a large percentage. The most marked effects appear in the eggs which have been transferred to sea-water after exposure to the $HgCl_2$.

There is little evidence of any modification in the development of eggs exposed to the solution for three minutes save in a slight increase in the length of time elapsing between insemination and the first cleavage. This does not markedly reduce the viability of the individuals over a long period of time, however, for most of them continue in their development and form subsequent stages at approximately the same rate as the controls. After 6 minutes' exposure, the results are essentially the same save that but 90 per cent of the eggs form blastulæ, a reduction of 6 per cent of the total number cleaving. The viability is good. Nine minute eggs show a marked effect which is evident first as the localized cytolysis described above, and later in the reduction of the percentage of cleaving eggs. Subsequently, further evidence of the solution's action is seen in the retardation of development and in the lowered viability of eggs in later cleavage stages. In many, development stops after the first, second, or third cleavage. The percentage of dead individuals in the cultures is continually increasing. As a result, relatively few reach even the early gastrula stage. These few show a marked retardation when compared with the control embryos or with those of cultures of 3 and 6 minutes' exposure.

The condition just described is much further developed in the 12-minute eggs. At least 10 per cent show a local cytolysis which involves a greater portion of the cortical region than is concerned in the 9-minute eggs. Cleavage is retarded far more and, when some of the eggs finally do cleave, the total number is but 20 per cent, whereas it was 96 per cent after 9 minutes' exposure. The lower percentage of the cleaving eggs is apparently related to an increase in the amount of the pigment clumping, which here shows the form pictured in Fig. 4, *a*, *c*, and *d*. Several eggs had cleaved in the cytoplasmic portion (cf. Fig. 4, *b*), a phenomenon much more frequently encountered in cultures of eggs exposed for longer intervals. It will be described in more detail below. In addition, some of the eggs show the isolation of the pigment as in Fig. 2. Examination of the culture after 24 hours showed but an occasional embryo still living, and in those development had been very atypical.

The analysis of material removed from the toxic solution after longer exposures is greatly complicated by the appearance of more of the type of egg shown in Fig. 4, *b* than were found in the 12-minute

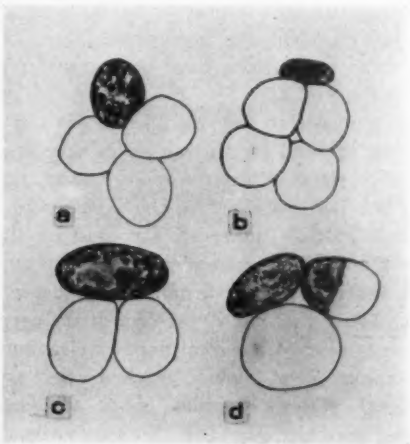

FIG. 5. Drawings to show the relation between the completely isolated pigment and the cytoplasmic fragmentation or cleavages which are found after exposures to more concentrated solutions of the salt. The cleavage in such cases does not involve the nucleus. For explanation see text.

group. In addition, eggs are frequent in which the pigment-free cytoplasmic portion is divided into even more units. Some of these are shown in Fig. 5, *a*, *b*, *c*, and *d*. It is always accompanied by the

accumulation of all of the pigment in one region of the egg. In the initial stages it looks much like a typical cleavage but, inasmuch as it is followed by coagulation and swelling and nuclear changes are not visible, it is evidently not a true cleavage but rather a purely cytoplasmic phenomenon. This conclusion is confirmed by the appearance of the eggs after fixation. The number of the eggs undergoing typical division falls abruptly. This may be seen in Table III. Many readings

TABLE III

Effect of M/40,000 $HgCl_2$ on rate and percentage of cleavage of eggs after various periods of exposure. Readings taken 227 minutes after insemination. Where the exposure is more than six minutes the viability of the eggs is poor.

Exposure *minutes*	Cleaved *per cent*	Extent of development
0	100	64-cell
3	98	64-cell
6	96	90% 64-cell
9	94	32-cell (many dead)
12	20	8-cell (cyt.)
15	4 (ca.)	Complicated by cleavages
18	0	as in
21	0	Fig. 4, *b*.

of the eggs have to be made to separate those with the cytoplasmic division from those showing true cleavage following rather extensive pigment localization. Approximately 50 per cent of the eggs show the cytoplasmic division. The majority of the remainder show the type of pigment localization pictured in Fig. 4, *c*, though there may be many more of the isolated pigment masses than are shown there. In the 10 per cent (ca.) of the eggs which eventually undergo a true cleavage the viability is very poor. As a result, less than one per cent go as far as the 32-cell stage and these die shortly afterward. Death is accompanied by swelling and evident coagulation of the cytoplasm.

M/35,000 $HgCl_2$.—Experiments with this concentration of the $HgCl_2$, when compared with those of less salt content, serve for the most part to demonstrate the increased toxicity of the solution on eggs after shorter periods of exposure. The solution was prepared in the same way as those above. Controls were set aside for future examination. Eggs were obtained from one female and sperm from one male. The insemination was made according to the formula, 5(1:25):50. The uninseminated control in sea-water showed no membrane elevation after 20 minutes while the uninseminated control in the $HgCl_2$ solution showed a little less than one per cent after but 7 minutes' exposure, a percentage which increased with the length of

exposure. The inseminated eggs showed 100 per cent membrane elevation at the end of 5 minutes, when transfer to the toxic solution took place. In the mercuric solution there was a noticeable widening of the cortical space between the fertilization membrane and the egg. The nucleus forms the monaster, but no further nuclear changes take place. As in previous cases, transfer to sea-water followed at 3-minute intervals. For the most part the results obtained in the experiment are directly comparable to those already described above, save that the action of the salt is evident after shorter exposure times. The greatest difference is seen in the occurrence of eggs with cytoplasmic division after as short an exposure as 18 minutes, and the relatively large number of eggs with multiple pigment loci. In this concentration, the effect is quite evidently one of cytoplasmic coagulation. The description of the first four groups of eggs, *i.e.*, those exposed for 3, 6, 9, and 12 minutes respectively deserves especial mention.

There is no localization of pigment in any of the eggs which have been in the $HgCl_2$ solution for but 3 minutes. One hundred per cent of the eggs cleave regularly, though in this experiment the cleavage takes place after a period of 53 minutes rather than 42 minutes after insemination, as in the controls. No other effects of the exposure appear up to the time of larva formation. The cleaving eggs go through the early developmental stages but slightly retarded, and form motile larvæ similar to the controls. This is not true of the eggs which have been exposed for 6 minutes. This group shows a marked localization of the pigment followed by extensive local cytolysis, (94 per cent of the eggs at 60 minutes). Quite a large amount of the cortical region of the egg is involved. The condition resembles that shown in Fig. 2. In spite of this 98 per cent of the eggs cleaved, though the cleavage did not start in any of the eggs for 63 minutes, 10 minutes later than division in the 3-minute culture, and 21 minutes later than the controls. The deleterious effects of the exposure are evident in each of the following exposures. Three and one-fourth hours after insemination, when the eggs of the control and the 3-minute group are in 16 and 32-cell stages, this culture shows not only 16-cell stages but also 2, 4, and 8-cell stages. Fifty per cent of the eggs are alive, while the rest have cleaved once, twice, or three times, where the development stopped. None of the eggs develop motile larvæ. It is quite evident, therefore, that the viability of the eggs has been markedly decreased by this short exposure to the action of the mercuric salt.

Quite in contrast to the rest of the experiments described, some few of the eggs which were in the 9-minute group show a pseudocleavage of the type pictured in Fig. 2. In no other case is this evident

after such short exposure. Localization of pigment takes place in all of the eggs after 60 minutes and in the majority is to be found in numerous foci (vid. supr.: multiple localization). The few eggs not showing either of these phenomena retained, theoretically at least, the possibility of dividing. No cleavage was observed in these 3 hours after insemination. Fifteen minutes later approximately 2 per cent of the eggs had divided. The viability of these few was so diminished, however, that none of them went beyond the 4-cell stage. The poisoning is complete in the 12-minute eggs, where no division takes place. This is probably intimately associated with the increase in the percentage of pseudo-cleavages and the marked extensive local cytolysis which takes place immediately upon the return of the eggs to the sea-water. All of the eggs eventually swell and cytolyze, though there is a marked differential effect in different eggs.

M/30,000 $HgCl_2$.—This experiment was set up in the same way as those described above. Uninseminated controls showed no membranes after 20 minutes when in sea-water, and over one per cent wide membranes when in $HgCl_2$ solution. One hundred per cent membranes were elevated on insemination and this was followed in sea-water by cleavage at 46 minutes. The temperature of the solutions at the initiation of the experiment was 22° C. There are a number of ways in which the eggs here differ from those described in former experiments, some of which involve merely an intensification of a previously noted action of the salt, while others introduce new phenomena. The first indication of the effect of the solution appears as a clumping of the eggs. This has not been noted in any of the previous experiments. There is evidently a modification of the quality of the surface membrane of such a nature that the eggs adhere to each other. Some modification or solution of the jelly layer investing the egg must be involved, for control eggs showed the jelly layer. This is followed by a pseudo-cleavage in the mercuric solution after 12 minutes' exposure. Such eggs cytolyze at the point of pigment accumulation after 3 to 6 minutes. After 30 minutes 80 per cent of the eggs show a cytolysis of the cortical region associated with an accumulation of pigment. The eggs transfered to sea-water show much the same sort of behavior. Even in eggs which are transferred after 3 minutes' exposure there is 100 per cent local cytolysis 63 minutes after insemination. Much of the cytolysis is very extensive. The pigment accumulation in the region of the cytolysis is a little greater than that described above for 6-minute eggs. Eggs which have been exposed to the mercuric solution for longer periods of time show further effects of the action of the salt. In many, coagulation appears very early, so that further cytoplasmic changes and

pigment migration stop, the eggs merely swelling and cytolyzing. Some of the most interesting modifications take place in the uninseminated eggs which were placed in the $HgCl_2$ solution. The pigment is clumped, but in a way visibly different from that in inseminated eggs. In addition, there is a cytoplasmic cleavage or fragmentation which is partial, involving one side of the egg only. This appears in but a very small percentage of the eggs. It may be associated with those in which membrane elevation has been produced by the solution, though no direct evidence on this point is available. No nuclear changes are involved.

In previous experiments nuclear changes proceed after inseminated eggs which have been in $HgCl_2$ solutions are returned to sea-water. It will be recalled that in the more concentrated solutions, the nucleus remains in the monaster stage during the time that the egg is in the toxic solution, further progress appearing only after retransfer. The nuclear changes in the solution of M/30,000 concentration proceed very slowly to the monaster stage, even this change occurring only in a small percentage of the eggs which are not greatly effected, after 18 minutes in the solution. When these are returned to sea-water, the changes proceed very slowly. Only those eggs which have been exposed for but 3 minutes show any advance over the monaster. These eggs are greatly retarded, showing only an early bar 27 minutes after insemination. The evidence of extensive injury is marked, therefore, not only in the behavior of the cytoplasm and its inclusions, but also in the nucleus. The depression of the viability of the eggs is so great in this experiment that none of the eggs divided, even when the exposure is as short as 3 minutes. This may be associated with the relation between nuclear and cytoplasmic phenomena.

Discussion

From the results obtained and presented above, we find that the mercuric chloride solution produces several specific changes in the egg, all of which vary directly with the concentration of the solution and the length of the exposure. A number of these phenomena deserve special consideration in the discussion. The "activating" influence of the $HgCl_2$ at certain concentrations will concern us first.

It has previously been reported (Lillie, 1921; Hoadley, 1923) that in relatively high concentrations of mercuric chloride, not only is the elevation of membranes wider on inseminated eggs than on inseminated sea-water controls, but uninseminated controls show a certain percentage of eggs with membranes. The percentage of uninseminated eggs elevating membranes on exposure to the solution varies directly with the concentration of the salt. The production of the membranes

is not immediate. They appear on certain eggs after as short an exposure as five minutes, in others the membranes do not elevate for a much greater period of time. There is no possibility of either hypotonic or hypertonic activation in any of the experiments cited. Although the subsequent development of eggs showing membranes elevated after exposure to mercuric chloride solutions has not been investigated, this must, I think, be interpreted as an initiation of development in the gametes. The evidence for this statement is derived entirely from close observation of the pigment behavior and the subsequent cytolysis of such eggs as compared first with that of membraneless eggs, and second with that of eggs with fertilization membranes elevated before exposure, both lots being left in the mercuric chloride solution during the observations. The eggs with membranes elevated after exposure to the $HgCl_2$ resemble the inseminated eggs in every way. There must be some alteration of the cortical membrane of the egg induced by the solution which is similar to that produced at fertilization, and which results in the elevation of a membrane and, subsequently, the like penetration of the mercuric ion. As a result, the reaction to the $HgCl_2$ is that of an inseminated rather than of an uninseminated egg.

As would be expected in the case of an extremely toxic solution, the effect is next evident in the cortical cytoplasm of the egg. The pigment granules are visibly affected first and most extensively. This in turn appears to be due to a specific mercury-avid property of the pigment itself. The degree to which the total amount of pigment in the egg is involved in this action is apparently of a quantitative nature as is evidenced by the experiments. Similar effects are produced by short exposure to concentrated solutions and longer exposures to less concentrated solutions. An indication of the extent of the action may be obtained by examining the behavior of the pigment subsequently accumulating at the point of the egg at which extrusion and local cytolysis occur. The very fact that after the extrusion of the pigment which is localized, as aforesaid, cleavage may take place and larvæ may be formed indicates that the cytoplasm as a whole is not greatly affected by short exposure to the mercury. Longer exposure has a more extensive effect, so that coagulation appears and the eggs are much damaged, further development not taking place. If, as would seem legitimate, one may regard the pigment as a mercury-avid substance, a conceivable mechanism is available by which the mercuric ion which has entered the egg may be bound and removed. Hence the egg is enabled to continue its development. The clumping of the pigment is very evidently not primarily a local response to the presence of the mercuric salt. Rather, as is evident in many of the experiments, the

pigment is equally affected throughout the entire cortical region of the cytoplasm. At a later time, the pigment becomes clumped or localized at some single point near the surface of the egg. Where the pigment clumps in a single mass, no coagulation of the egg cytoplasm appears, as is the case on exposure for longer periods of time or to more concentrated solutions. Where coagulation takes place before the migration of the pigment is complete, there are a number of small foci, isolated one from the other. Apparently the migration of the pigment has been arrested by the coagulation of the surrounding cytoplasm. It is also interesting to note that in the majority of cases where coagulation does not occur, cleavage may follow the expulsion of the single pigment focus. There is no evidence of any correlation between the site of pigment accumulation and the original polarity of the egg in *Arbacia*. It is possible that an examination of the behavior of other forms may yield valuable information concerning this point.

The elimination of the pigment in the eggs and their continued development is of interest from yet another standpoint. Pigment in eggs has been regarded as of developmental significance by many workers. By some it has been thought to be of importance because of its association with oxidative processes within the germ. Warburg (1914), in experiments with *Strongylocentrotus lividus,* found that oxidations in the egg were associated with the granular portion. We might expect that if oxidative processes are intimately associated with the granular portion of the egg, and hence, in part at least, with the pigment granules, loss of the pigment granules would have a definite effect on the future development of the egg. It has already been shown by a number of workers that oxidative processes and developmental rate over short periods of time are discrete in their action. This is emphasized by Whitaker (1929) in a report of observations on the relative rate of development in pigmented and unpigmented fragments of *Arbacia* eggs. In the experiments reported above there is an evident retardation in the developmental rate and also a marked reduction of the viability of the gametes. In view of Whitaker's results, however, we must look in another direction for the interpretation of the facts.

The retardation in developmental rate is evident in all cases in which eggs are exposed to mercuric chloride solutions. The extent of both the slowing down of the cleavage rate and the loss of viability is directly dependent on the strength of the solution and the length of the exposure. Apparently it is more extensive after pigment has been extruded than when it has not, but this is always attendant on a longer exposure period or a greater concentration of the solution. There is a

perceptible lag of the effect on the viability over the effect on the rate of development. They show a correlation in that they both increase with an increase in the effective period of the exposure. Eventually the interpretation of the two is confused by the fact that only one or two cleavages take place, the individuals then dying. This is especially marked after even short exposures to high concentrations of the salt. The amount of the pigment extruded in such cases is not very much greater than that at lesser concentrations, and besides, we are dealing with the early stages in development when oxidation rate and developmental rate are quite independent. For that reason it appears that the mercuric ion, both in retarding development and in lowering the viability, acts directly on the cytoplasm of the egg rather than indirectly, through the medium of the pigment granules.

Two types of cytolysis are associated with the action of the mercury on the egg. In all cases where the eggs are allowed to remain in the solution, they eventually coagulate and swell, no cleavage taking place. The picture in the eggs which are retransferred to sea-water is quite different. There, the pigment accumulates and the cytolysis which follows involves only the region of pigment clumping and extrusion. The rest of the cytoplasmic portion of the egg is little affected, as has been mentioned above, so that cleavage and larva-formation follow. The phenomena involved in the local cytolysis associated with the pigment elimination lead to the conclusion that the product of the mercury bound pigment is responsible for this action. The mechanism involved is not clear.

The results of the investigation may be briefly summarized as follows:

1. Mercury has an effect upon the egg of the sea-urchin *Arbacia* which is unlike that found in the case of any of the other metallic chlorides investigated.

2. Acting first on the cortical region it activates membrane elevation.

3. After longer exposures it has a direct effect upon the pigment which has mercury-avid properties. The pigment reacts to the mercuric solution by accumulation and subsequent extrusion at a localized point (or points) on the surface of the egg.

4. The extrusion of the pigment is accompanied by a cytolysis of the pigment granules and the associated cytoplasm.

5. The development of the zygote is retarded and its viability is lowered by the action of the mercuric solution.

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THE CLEAVAGE OF POLAR AND ANTIPOLAR HALVES OF THE EGG OF CHÆTOPTERUS

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The following experiment was carried out as part of a program to study the phenomenon of yolk-lobe formation that occurs in the eggs of certain annelids and molluscs. This peculiar phenomenon simulates cell-division to a striking degree, yet at the final moment, when the yolk-lobe is attached only by its stalk to one of the cells, a reversed reaction takes place and the material of the lobe is absorbed by the egg, or by the blastomere to which it is attached.

There were two relations that we wished to examine by cutting the egg in two: First, to find out whether the polar lobe develops on both fragments or only on one of them; second, whether the unequal first cleavage is dependent on or the result of the presence of an antipolar yolk field, or independent of its presence. In addition there were one or two other questions that we hoped to clear up: *e.g.*, whether there is any relation between the appearance of the pear-shaped form that the undivided egg assumes just prior to the appearance of the yolk-lobe and the mitotic phenomenon; whether the surface of the egg in the region of the constriction that produces the yolk-lobe changes before or during the formation of the lobe; and whether the condition of the mitotic figure at the moment of the lobe's formation bears any causal relation to the formation of the lobe. The experiments were made in the summer of 1928 at Woods Hole.

Eggs taken from parapodia were put into sea water. The germinal vesicle breaks down in a few minutes, and a spindle, pointing to the pole, is formed. The polar region can be made out owing to the clear region around the spindle. The egg remains in this condition until fertilized. Just before cleavage the egg becomes pear-shaped with the apex at the pole. A little later the egg begins to bulge at the antipole to form the yolk-lobe that does not constrict off from this egg as markedly as it does in some other eggs, such as *Ilyanassa* and *Dentalium*. Sections of the preserved egg during these periods show that the pear-shaped stage first appears when the two pronuclei have come together and the walls are disappearing. The two asters of the future division-

figure are present and well developed. Just what internal condition leads to the change in shape is not shown from these relations. One might surmise, however, that the collapse of the very large pronuclei with the resulting distribution of their fluid contents, or possibly the nearer approach of the two astral fields resulting from the collapse of the nuclei, may be connected with the change in shape of the whole egg.

Almost immediately the egg becomes rounded again and begins to elongate in a direction at right angles to the plane of the oncoming first division. At this time a bulging around the antipole indicates the development there of the antipolar lobe. As the cleavage proceeds the lobe becomes more conspicuous, and later becomes constricted at the point of contact with the egg. The constriction becomes deeper, giving the lobe an oblong or even rounded appearance, but in *Chætopterus* the constriction is never carried as far as in the eggs of some molluscs. At this time the cleavage furrow is progressing, but, from the first, does not give so much the appearance of cutting through the lobe as in *Dentalium*. As the furrow deepens it passes to one side of the lobe. The point of attachment of the lobe remains on the larger or CD-blastomere.

Sections of eggs that have been preserved in picro-formalin, or Flemming's osmic acetic, stained in iron hæmatoxylin and counterstained in erythrocene, do not reveal any unusual changes in the antipolar region during this period. Some of the yolk (and its surrounding protoplasm) simply protrudes into the lobe. This yolk is a part of the cup-shaped mass that lies over the lower hemisphere with the edges of the cup extending toward the polar field. The yolk that goes into the lobe is not discontinuous with the rest of the material. One gets the impression that it is squeezed into the protrusion or bulges into it as the lobe develops. As the base gradually constricts there is, in sections, visible in the superficial layers nothing that is peculiar or different from the rest of the neighboring surface. The impression that one gets from sections is that the rounding up of the materials that become the two blastomeres does not include the antipolar field, and that the lobe is a by-product, so to speak, of these changes, and not in itself actively engaged in the process of its formation.

This interpretation may appear at first sight to be in contradiction to the observation that Wilson has made of the behavior of the yolk-lobe of *Dentalium* when severed from the CD-blastomere. He found that the isolated lobe showed alternate periods of rest and activity that were synchronous with those of the next two divisions of the egg when the yolk-lobe reappeared. In some cases he observed indications that the yolk-lobe itself formed a lobe. These observations may not

appear to harmonize with the supposition offered above, that the lobe, as such, is a passive factor in the result, and its development the resultant of the mitotic constrictions about the division centres; but if, as is not impossible, a rhythmical impetus, or something of the sort, is initiated in the cytoplasm, it might conceivably be supposed to affect even the isolated lobe, or even involve the formation of a cytoplasmic aster in the lobe. Until observations are made on the interior of the isolated lobe at the time of its activities we can only speculate as to the causes of this remarkable phenomenon.

The operations on the egg were made before fertilization. The outer membrane is very tough, even before fertilization, so that it is difficult to sever it completely without destroying the egg within. This difficulty is increased by the tendency of the *Chætopterus* egg to burst, and care is necessary in cutting in order to avoid great injury. It can be done, however, with a quartz needle and the micro-dissection instrument. The egg is least injured if slowly pinched apart, and if the membrane is gently twisted by rolling the needle back and forth. Damage to the fragments is further reduced if the outer membrane is not entirely severed after the egg is well cut apart. The results obtained from a few eggs with membranes entirely severed were the same as those obtained from the eggs whose fragments remained in the same membrane. It does not seem likely that the membrane connection between the fragments has any effect. Independent fertilization of both fragments takes place. All of the operations here described were made either in the equatorial plane or parallel to it.

The polar fragment becomes pear-shaped at about the time after fertilization when the egg passes through this stage. It then elongates for the first cleavage, but a yolk-lobe does not appear at the antipolar surface of the fragment. It is true that sometimes a bulging, or other irregularity, appears in the region where the cut was made, especially in the fragments most damaged in cutting, but it does not have the distinctive shape of the normal lobe. It seems rather to be due to some weakness at the cut surface, and as a result, perhaps, of changes of tension within the egg or at the surface.

The first cleavage of the polar fragment is into unequal parts, which, in general, have the same relative size as have the first two cells of the normal egg. Since the yolk region has been removed. and still the unequal cleavage appears in the fragment, the inequality cannot be explained in the normal egg as due to the presence of yolk or yolk-lobe material in the antipolar hemisphere.

The antipolar half of the egg, which contains only a sperm-nucleus, does not assume a pear-shape prior to the first cleavage, but at the

time when the cleavage is about to begin a typical antipolar lobe appears. The size of the lobe is approximately proportionate to the size of the fragment. If at the time of the operation all of the material that normally goes into the antipolar lobe is already present in the antipolar hemisphere, it would appear that the formation of the antipolar lobe is not simply the extrusion of a given amount of inert material around the antipole, but is correlated with the size of the dividing materials and possibly with the size of the astral spheres.

The first cleavage is into unequal cells. The antipolar lobe comes to lie in the larger cell into which it is later absorbed. Whether this difference in size of the first two cells is in the same ratio as in the polar fragment cannot be stated positively, but there is certainly no striking difference in the two cases. Here, again, it may be pointed out that the unequal cleavage is not dependent on the amount of yolk in the dividing cell. Were it so, the smaller cell might be expected to be disproportionately smaller in the basal fragment.

These general statements may be supplemented by the following numerical data. In 19 cases both halves developed and conformed to the above description. In 3 cases the polar half conformed; the antipolar was somewhat abnormal. In 3 cases both approximately conformed but were somewhat abnormal. In 5 cases the polar conformed, the anti-polar did not develop; in one case neither developed. In addition there were two cases in which the antipolar lobe was not seen on the basal half, and in one case the antipolar half formed a lobe but did not divide; the polar half failed to develop.

Mead ('98) described the early cleavage stage of the egg of *Chætopterus* with numerous drawings of the mitotic figure during cleavage. At the time when the yolk-lobe appears, the " rays " from the astrosphere of the CD-cell are represented as extending into the polar lobe. The antipolar cleavage plane is represented as lying at the crossing-point of the rays of the two blastomeres to one side of the lobe. From the figures, which appear partly schematized, it is not certain whether these rays are at their outer ends anything more than lines resulting from the arrangement of the peripheral yolk granules.

Lillie ('06) has also described the early cleavage stages of the *Chætopterus* egg. He found that after centrifuging the yolk is driven away from the antipolar field in some of the eggs that have fallen in the centrifuge with the polar hemisphere turned outward. When these eggs cleave the yolk-lobe may contain the oil field, or the clear middle zone, proving that the yolk as such is not essential to the formation of the polar lobe. The more superficial layers of the egg are little, if at all, distributed by the centrifuging, and what is here more to

the point, the mitotic figure occupies the normal position in such eggs with respect to the pole and to the antipolar field.

Wilson ('29) has recently described the cleavage and development of egg-fragments of the *Chætopterus* obtained by centrifuging. The eggs, for the most part, seem to fall at random in the centrifuge tube, and are stratified without regard to the polar axis—at least, all possible relations may be found. Strong centrifuging causes the eggs to elongate and often the clear (centripetal) end constricts off from the yolk-bearing end. The nucleated fragment, that lies nearer to the pole, can be identified because such a fragment gives off the polar bodies. Unless it could be shown that the spindle is also displaced at times by the amount of centrifuging here used, this result makes the identification of the polar fragment certain, regardless of whether it contains the yolk or the oil cap. After fertilization both fragments may cleave. Wilson finds that the unequal first and second cleavages are characteristic of both fragments. In general, only those fragments that do not give off polar bodies develop a yolk-lobe. Our own results confirm entirely these conclusions. Their only merit is that they give, perhaps, more accurate information regarding the regional origin of the fragments, and make possible comparisons between fragments of equal sizes of the same egg whose interior has not been disturbed by centrifuging.

If, then, as these experiments appear to indicate, the antipolar lobe-formation is not an essential part of the cleavage pattern but a by-product of that pattern, its absence from the polar fragment and its presence in the antipolar fragment remains to be explained. It is reasonably certain from Lillie's and Wilson's centrifuging experiments that this lobe is not directly caused by the presence of a particular kind of yolk material at the antipole, but the occurrence of this material might, by influencing the location of the mitotic figure, determine the extra-territorial region that becomes the lobe. If so, the relatively greater development of the mitotic figure in the polar fragment might in itself account for the absence in it of a lobe at the two-cell stage. Conversely for the antipolar fragment. But there is an alternative possibility, namely, the relative location (and size) of the first spindles in the two cases. If, for instance, it could be shown that in the polar fragment the spindle is relatively nearer the centre and in the antipolar fragment relatively nearer the polar side of the fragment, the two results would be in accord with the hypothesis suggested above.

THE ABSORPTION SPECTRA OF SOME BLOODS AND SOLUTIONS CONTAINING HEMOCYANIN

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The present study of the absorption of light by hemocyanin was undertaken in the course of developing a spectrophotometric method for the determination of the quantity of oxygen combined by the blood of invertebrates which contain this pigment. The data obtained provide a precise description of the color characteristic of the body fluids of the various animals examined. Attention has been directed not only to the spectrum of oxygenated blood, which has already been examined with precision in the case of a number of organisms by Dhéré and his collaborators (1919, 1920, 1929), Begemann (1924) and Quagliariello (1922), but also to the apparent absorption of light by reduced blood. The latter observations have led to the conclusion that a very considerable fraction of the light passing through a hemocyanin solution may be scattered by the hemocyanin molecules. The extent of this scattering determines in large part the color characteristic of the various bloods when examined either by reflected or transmitted light. By taking account of the amount of light scattered by the reduced solution, it has been found possible to determine the characteristic absorption spectrum of the molecular complex responsible for the bluish color developed when the hemocyanins combine with oxygen. In this way some information is obtained on the specificity of the oxygen-combining mechanism in the blood of different animals.

METHOD

Observations have been made upon the blood of the conch, *Busycon canaliculatum,* the horse-shoe crab, *Limulus polyphemus,* the squid, *Loligo pealei,* and the lobster, *Homarus americanus.* The bloods have been drawn by methods previously described (Redfield, Coolidge and Hurd, 1926), and preserved in the cold with toluene until prepared for observation. Under these conditions they may be kept with little change for many days. The bloods have been diluted to concentrations appropriate for the methods involved with sea water, distilled water, or various salt solutions, after which they have been allowed to stand

overnight in the ice box in order to permit equilibrium with the modified environment to be reached. The material has then been filtered and placed in specially constructed tonometers in which it could be brought into equilibrium with various mixtures of gases. Each tonometer consisted of a cylindrical pyrex glass bottle of 200 cc. capacity, to the bottom of which a T-tube was sealed. The ends of the T were ground parallel to one another and were closed with optically flat glass plates sealed in position with DeKhotinsky cement. A chamber was thus provided, having an inside diameter of approximately one centimeter and a length which was in most cases exactly 3.3 centimeters. Following equilibration with the gas mixture, the sample of solution could be run down into the T-tube and the intensity of the light transmitted through it, measured. The specimens were oxygenated by filling the tonometer with oxygen or, in those cases where the character of the oxygen dissociation curve permitted, with air. Solutions containing reduced hemocyanin were prepared by evacuating the bottles after the introduction of the solution and refilling them with hydrogen. The bottles were then rotated for 15 minutes, after which the bottles were re-evacuated and again filled with hydrogen and equilibrated for an additional period of 25 minutes. The precision of the measurements is affected if the solutions are not perfectly clear. For this reason, the greatest care is necessary in filtering the solutions and in being sure that the dissolved materials are in equilibrium with their environment before filtration occurs, as otherwise small amounts of precipitated material may appear in the solutions before the photometric measurements are made. Reduced hemocyanin solutions are particularly troublesome because small amounts of material become insoluble during the mechanical disturbances incidental to evacuation and equilibration of the solutions. Under favorable circumstances, the insoluble particles produced in this way settle out if the specimens are allowed to stand for an hour or more prior to making the measurements. Under other circumstances the solutions remain slightly cloudy and the precision of the measurements is seriously interfered with. The second difficulty is in obtaining complete reduction of the solutions. Reduction appears to be satisfactorily attained by the method outlined above in the case of the bloods. In solutions of purified hemocyanin, because of the change in the shape and position of the oxygen dissociation curve, complete reduction is much more difficult to obtain. Further repetition of the processes of evacuation and equilibration with hydrogen would undoubtedly achieve the desired effect, but unfortunately such repetition increases the amount of insoluble material formed in such solutions and thus defeats its purpose. The use of chemical reducing agents has

not been employed as those which have been tried have led to progressive changes in the color of the reduced material, which again defeats the objects of the experiments.

Measurements of the absorption of light by these solutions have been made with a König-Martens spectrophotometer constructed by Schmidt and Haensch. The light source of the instrument was illuminated by a Mazda projection bulb, the intensity of whose light could be controlled by a rheostat. The width of the slits was kept at 0.2 millimeters except at wave lengths less than 480 mμ, when it was increased to 0.4 or 0.6 millimeters as required in order to secure sufficient illumination. The calibration of the wave length scale of the instrument was checked from time to time and was found at all times to be accurate within 1 mμ. The precision of the instrument was also checked by the determination of the absorption of two colored glass filters, which had been standardized by the U. S. Bureau of Standards.

The absorption of light is indicated by the following equation:

$$\frac{I_0}{I} = \frac{\tan^2 a_0}{\tan^2 a_1}, \tag{1}$$

where I_0 is the intensity of incident light, I the intensity of transmitted light, a_1 the angle of the analyzing prism at which the fields match when a tube containing the solvent is placed in one of the beams of light; a_0 is this angle when the tube containing the solution is placed in this beam. In all cases, a_1 was determined with the absorption vessel filled with distilled water. Test showed that the result was the same, within the limits of observational error, in whichever beam the absorbing solutions were placed. In order to obtain results which might be compared with one another after the blood of different animals was examined, the results have been expressed in terms of the extinction coefficient, E, characteristic of each wave length as defined by

$$\frac{I}{I_0} = 10^{-Ed} \tag{2}$$

where d is the length in centimeters of the column of fluid. It follows that the extinction coefficient, E, is given by:

$$\frac{2(\log \tan a_0 - \log \tan a_1)}{d}. \tag{3}$$

In dealing with the absorption of light by hemocyanin, one is concerned particularly with the absorption of light by the complex formed when oxygen unites with hemocyanin. In this union it has been demonstrated that one atom of oxygen is combined for each atom of copper contained

in the hemocyanin. The union appears to depend upon some grouping in the hemocyanin molecule, of which the copper forms an essential part. For convenience we will refer to this arrangement as the "chromatic group." For purposes of comparison it is interesting to determine the absorption of light in relation to the number of chromatic groups present. According to Beer's Law, the extinction coefficient of a substance in solution is proportional to its concentration. We have consequently expressed the absorption of light by the hemocyanin solutions in terms of E/c, where c is the concentration of copper in the solution expressed as milligram atoms per liter. An advantage of this notation also lies in the fact that the concentration of copper in serum may be readily obtained without the necessity of determining the number of grams of hemocyanin which are present, an investigation which cannot be made unless the hemocyanin of the species has been isolated and properly studied.

The Application of Beer's Law to Hemocyanin Solutions

The foregoing treatment assumes explicitly that in the absorption of light by hemocyanin solutions Beer's Law is valid and that in consequence E/c is a constant characteristic of the substance at each wave

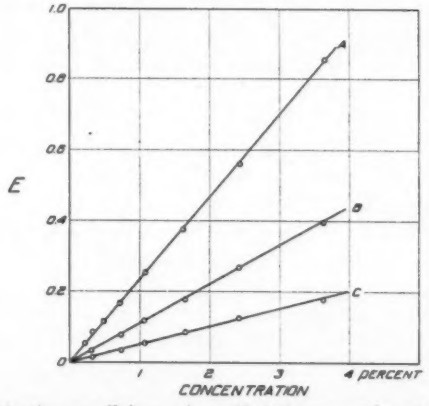

Fig. 1. Extinction coefficient of purified hemocyanin solutions of various concentrations.
A. Busycon canaliculatum at 570 mμ.
B. Limulus polyphemus at 580 mμ.
C. Limulus polyphemus at 480 mμ.

length. Quagliariello (1922) and Svedberg and Heyroth (1929) both present evidence that Beer's Law does not apply in the case of hemocyanin solutions. We have consequently examined this question care-

fully and have found no indication that Beer's Law is not valid when applied to such solutions and to such concentrations and at such wave lengths as we have employed. In Fig. 1 is shown the relation between the extinction coefficient of solutions of purified hemocyanin of two species made at various concentrations. In the case of *Busycon* and of *Limulus* the measurements were made at the wave length of maximal absorption and in the case of *Limulus* also at the wave length at which the absorption is minimal. In all three cases the relation between extinction coefficient and concentration is linear within the accuracy obtainable with photometric measurements on solutions of this

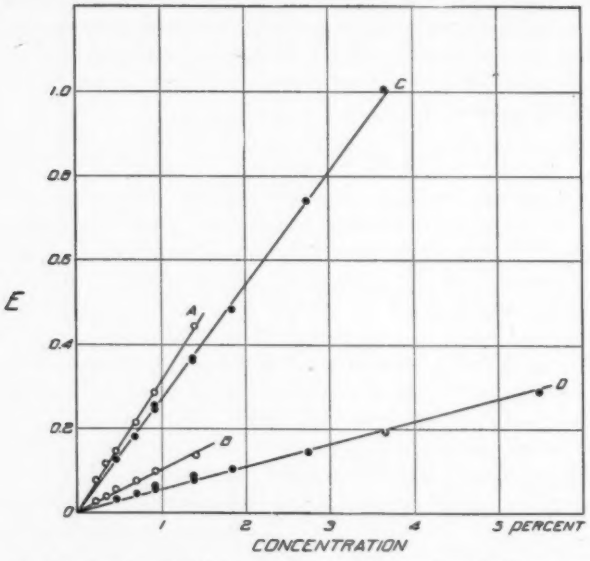

FIG. 2. Extinction coefficient of oxygenated and reduced solutions containing hemocyanin at various concentrations.

A. Busycon canaliculatum serum oxygenated. Dilution with 2.5 per cent NaCl. Wave length 570 mμ.

B. The same, reduced.

C. Busycon canaliculatum hemocyanin in potassium phosphate buffer solution oxygenated. Wave length 570 mμ. Dilution with phosphate buffer, 0.178 molecular phosphate; ionic strength 0.55; molecular fraction as K_2HPO_4 0.90.

D. The same, reduced.

character. Quagliariello's measurements were made upon native blood diluted with 2.5 per cent sodium chloride. It seemed possible that his anomolous results were due to alterations in the environment of the hemocyanin as the result of dilution, which might possibly affect the

degree of scattering of light by the protein, to be subsequently discussed. We have therefore made observations on the serum of *Busycon canaliculatum* similarly diluted with 2.5 per cent sodium chloride and have measured the extinction coefficient not only of the oxygenated but of

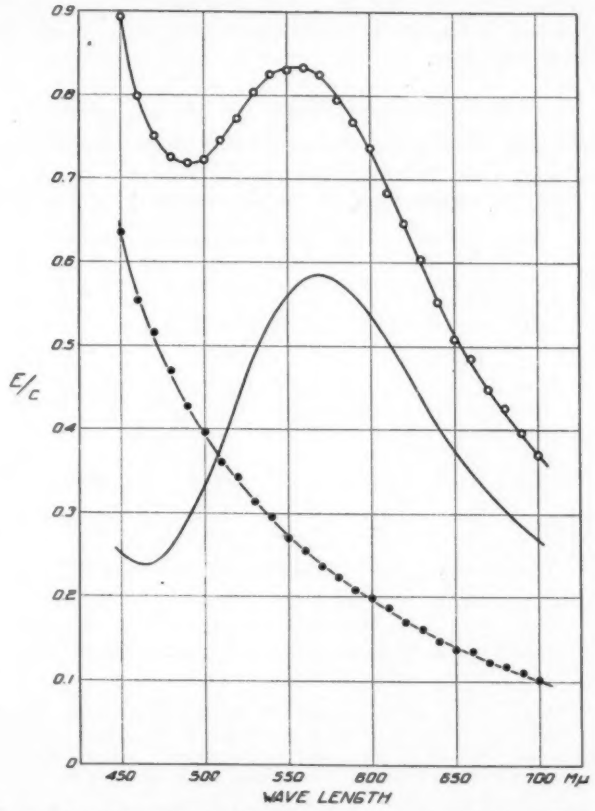

FIG. 3. Absorption spectra of blood of *Busycon canaliculatum*. Upper curve, oxygenated blood; lower curve, reduced blood; intermediate curve, corrected spectrum of chromatic group. Copper content of blood 0.066 mgm. per cc.; dilution, 10 parts blood plus 18 parts H_2O plus 2 parts 0.1N NaOH; pH 9.6; length of absorption vessel 3.3 cm.

the reduced solutions. The results are shown in Fig. 2, curves *A* and *B*. Again it appears that the relation between extinction coefficient and concentration is practically linear. As a further test we have made observations upon a solution of purified *Busycon canaliculatum* hemo-

cyanin dissolved in potassium phosphate buffer and diluted carefully with a similarly buffered solution so as to maintain constant ionic strength. Measurements were made upon both the oxygenated and reduced solutions which again conform closely to the requirements of Beer's Law (Fig. 2, *C* and *D*). We consequently conclude that the assumption of Beer's Law is valid in connection with the observations discussed in this paper.

The Absorption Spectra of Native Blood

The typical spectra of the oxygenated and reduced bloods of *Limulus, Loligo, Busycon* and *Homarus* are presented in Figs. 3, 4, 5 and 6. Detailed descriptions of the solutions will be found in the

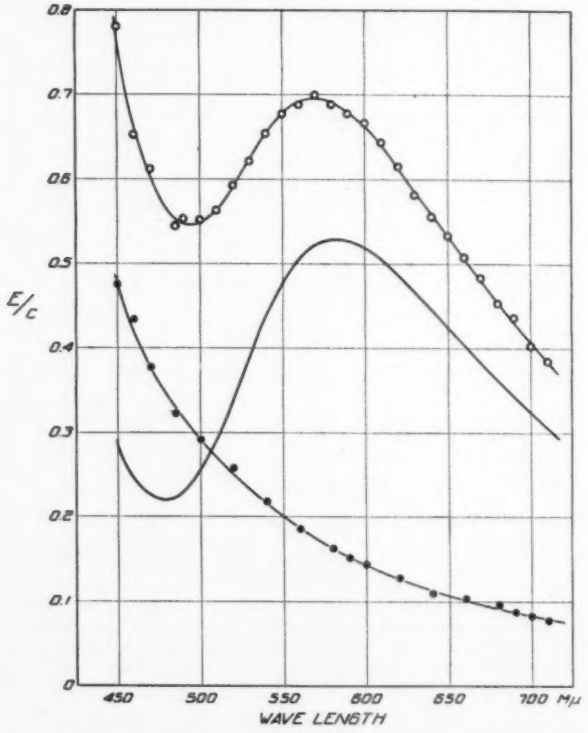

Fig. 4. Absorption spectra of blood of *Limulus polyphemus*. Upper curve, oxygenated blood; lower curve, reduced blood; intermediate curve, corrected spectrum of chromatic group. Copper content of blood 0.081 mgm. per cc.; dilution, 20 parts blood plus 35 parts sea water plus 5 parts 0.08N HCl; pH 6.05; length of absorption vessel 3.3 cm.

legends of these figures. The upper curve in each case represents the absorption of light by the oxygenated blood, the lower curve by the reduced solution. A glance at the curves descriptive of the oxygenated blood serves to show a very considerable difference in the shape of each curve and in the general magnitude of the absorption. The curves do not differ markedly from those described by Quagliariello and others

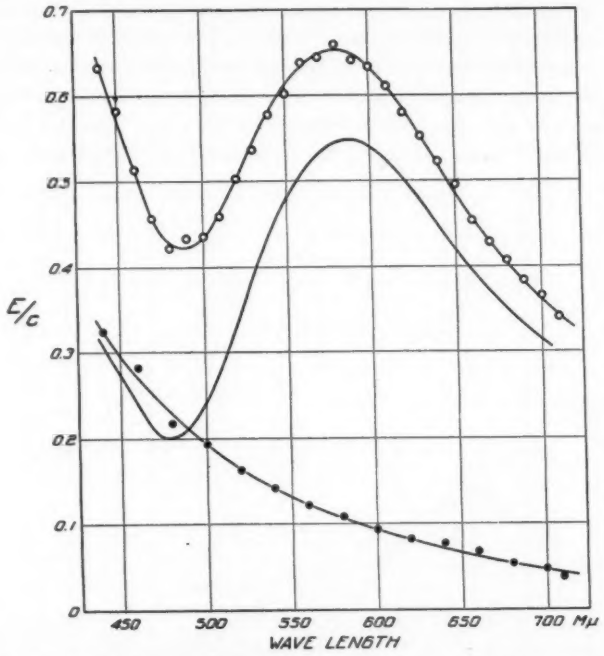

FIG. 5. Absorption spectrum of blood of *Loligo pealei*. Upper curve, oxygenated blood; lower curve, reduced blood; intermediate curve, corrected spectrum of chromatic group. Copper content of blood 0.249 mgm. per cc.; dilution, one part blood plus 6 parts sea water; pH 8.11; length of absorption vessel 3.3 cm.

in the case of European forms belonging to related groups. The curves are alike in displaying a broad band of maximal absorption in the yellow with more or less increased transmission in the region of blue-green. It is in the relative values of the absorption in the blue-green and in the yellow regions that the curves differ characteristically, the species falling in the order *Busycon, Limulus, Loligo, Homarus* as the absorption in the blue-green region decreases. It is, of course, this difference which determines the observed colors of the different bloods.

Spectra of Reduced Bloods

The spectra of the reduced bloods described by the lower curves in Figs. 3, 4, 5 and 6 deserve particular attention. It may be noted in each case, except that of the lobster, that these curves are similar in sweeping with gradual ascent uninterrupted by any obvious absorption bands as one passes from longer to shorter wave lengths. Comparing these curves for the different species, it may be noted that the absorption of light by the reduced blood is greatest in those forms in which the absorption by the oxygenated solution at the blue end of the spectrum is relatively high, the order being again *Busycon, Limulus, Loligo*. This fact may also be related to the observation of Redfield, Coolidge and Hurd (1926) that the Tyndall effect of the bloods studied

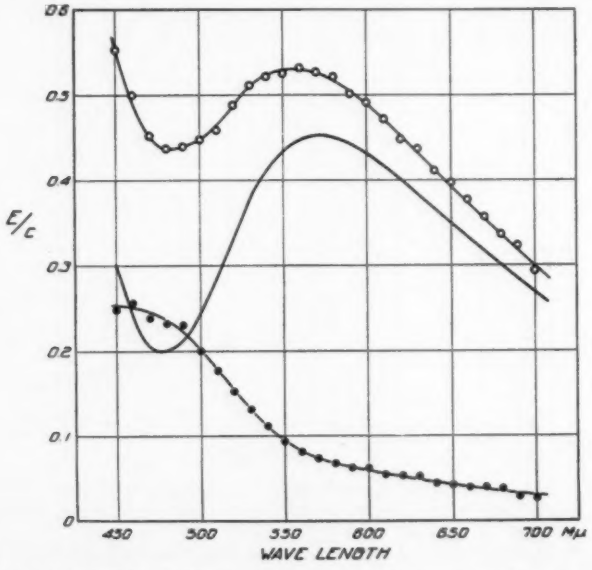

Fig. 6. Absorption spectrum of blood of *Homarus americanus* containing natural pigments. Upper curve, oxygenated blood; lower curve, reduced blood; intermediate curve, corrected spectrum of chromatic group. Copper content of blood 0.0505 mgm. per cc.; dilution, 2 parts of blood plus one part of solution containing 0.4 mols NaCl, 0.01 mols KCl, 0.02 mols $CaCl_2$ per liter; pH 7.87; length of absorption vessel 3.3 cm.

by them decreases in the order *Busycon, Limulus, Loligo* and suggests that the absorption of light by reduced bloods may be due almost entirely to the scattering of light by the solution. The absence of

definite absorption bands in the reduced blood of these three species supports this hypothesis.

According to Lord Rayleigh (Strutt, 1871), when a beam of light passes through a medium containing particles small when compared with the wave length, the light of various wave lengths is scattered in proportion to the reciprocal of the fourth power of the wave length. The light, which is scattered at an angle of 90° from the incident beam, may be expected to be completely polarized provided the particles are spherical. Observation of the Tyndall beam emitted by hemocyanin solutions shows indeed that the Tyndall light is polarized, and inasmuch

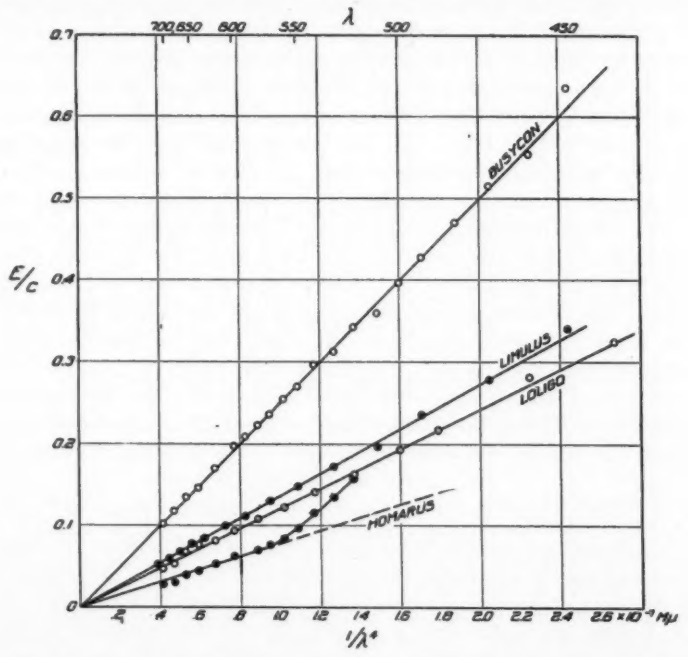

FIG. 7. Extinction coefficients, E/c, of reduced blood plotted against the reciprocal of the fourth power of the wave length, $1/\lambda^4$. For data regarding *Busycon*, *Loligo* and *Homarus* see legends to Figs. 3, 5 and 6. The data for *Limulus* is presented under Fig. 11 at pH 8.77. Concentrations, c, are expressed as milligram atoms of copper per liter.

as the beam disappears entirely when viewed through a properly oriented Nicol prism, the polarization must be very nearly complete. Rayleigh deduces that the attenuation undergone by the beam as the result of

scattering can be expressed by the equation

$$I = I_0 e^{-Kx\lambda^{-4}}, \tag{4}$$

where x is the thickness of the scattering medium, λ is the wave length, and K is a constant characteristic of the solution in question. The validity of this equation was demonstrated in the case of mastic solutions by Abney and Festing (1886). Mecklenburg (1915) has shown that solutions of colloidal sulfur scatter light in proportion to the reciprocal of the fourth power of the wave length when the diameter of the particles falls between 5 and 93 mμ. For larger particles the relation no longer holds. The radius of the molecules of hemocyanin of *Helix* and *Limulus,* according to Svedberg and Heyroth (1929), are of the order of 10^{-6} centimeters or 10 mμ, so that we may expect the Rayleigh equation to apply in their case. From inspection of equations 2 and 4, it is obvious that for any given solution E or E/c should be proportional to $1/\lambda^4$. We may consequently test the hypothesis that the apparent absorption of light by bloods containing reduced hemocyanin is due to the scattering of light by the hemocyanin molecules by determining whether E/c at each wave length is proportional to the reciprocal of the fourth power of the wave length. In Fig. 7 the values of E/c for the various reduced bloods are plotted against $1/\lambda^4$. The lines so formed in the case of *Busycon, Limulus* and *Loligo* are straight lines which on exterpolation converge toward and meet at the origin, indicating that the Rayleigh formula does in effect describe the phenomena observed. It may be concluded consequently that the apparent absorption of light by the reduced blood of *Busycon, Limulus* and *Loligo* is to be attributed to the scattering of light by the dissolved hemocyanin.

The Corrected Spectra of the Chromatic Groups

The absorption of light by oxygenated blood must now be attributed to at least two components: the apparent absorption due to scattering and the true absorption due to the chromatic group. If these are the only factors involved, and if it be assumed that the scattering of light by the hemocyanin molecule is unaltered by the process of oxygenation, it is possible to correct the absorption spectra of the oxygenated bloods for the apparent absorption due to scattering and obtain a corrected spectrum of the chromatic group itself. If the attenuation undergone by the beam of light as the result of scattering is given by

$$\frac{I_1}{I_0} = 10^{-E_r d},$$

where I_1 is the intensity of "unscattered" light which would emerge

were no other factors involved, and E_r is the extinction coefficient characteristic of the reduced material; and the further attenuation due to absorption by the chromatic groups is indicated by

$$\frac{I_2}{I_1} = 10^{-E_x d}$$

where I_2 is the final intensity of the emerged beam and E_x is the extinction coefficient expressing the effect of the chromatic group, then

$$\frac{I_2}{I_0} = 10^{-(E_x + E_r)d}.$$

The total absorption of light, however, is given by

$$\frac{I_2}{I_0} = 10^{-E_0 d}$$

where E_0 is the extinction coefficient of the oxygenated solution. Consequently,

$$E_0 = E_x + E_r.$$

The extinction coefficient of the chromatic group at unit concentration is consequently obtained by subtracting the value of E/c for the reduced solution from the value of E/c for the oxygenated solution at each wave length. This has been done, and the results are indicated by the intermediate curves in Figs. 3, 4, 5 and 6.

The Spectra of Blood Containing Other Pigments

The blood of the lobster requires special consideration because in addition to hemocyanin, this blood, in common with that of other crustaceans, contains the pigment tetronerythrin described by Halliburton (1885). Consequently the reduced blood of this species usually has a pinkish color and the bluish hue of the oxygenated blood has a more neutral color than that of the other forms if the pigment is present in sufficient amounts. As the result of the presence of this pigment, the spectrum of reduced lobster blood does not conform to the Rayleigh equation, as the lower curve in Fig. 7 shows. The tetronerythrin may be extracted from the blood by shaking the blood with chloroform. In Fig. 8 the absorption spectrum of the pigment extracted with chloroform is illustrated, the absorption of the dissolved pigment being compared with the absorption when the vessel is filled with chloroform. This substance possesses a maximal absorption at a wave length of 490 mμ and transmits nearly all of the incident light at wave lengths greater than 600 mμ. The apparent absorption of light due to scattering by the reduced blood of the lobster may consequently be arrived at

approximately. By considering the absorption spectrum of the reduced blood at wave lengths greater than 600 mμ, it may be observed from Fig. 7 that these points fall along a straight line drawn from the origin of the diagram. Extending this line beyond 600 mμ indicates the degree of apparent absorption due to scattering at these wave lengths.

The presence of tetronerythrin or similar pigments, the color of which is unaffected by the oxygenation of the blood, does not interfere

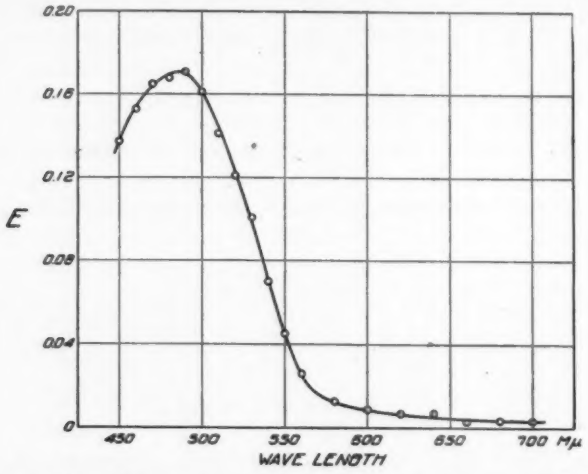

FIG. 8. Absorption spectrum of solution of the pigment extracted from lobster blood with chloroform. Concentration unknown; length of absorption vessel 3.3 cm.

with the determination of the corrected spectrum of the chromatic group. This may be demonstrated by examining the spectrum of blood from which the tetronerythrin has been extracted by chloroform. The spectra of oxygenated and reduced lobster blood so treated are illustrated in Fig. 9. It may be observed that the spectrum of the reduced solution no longer shows the irregularity due to the pigment. The corrected spectrum of the chromatic group may be seen to be almost identical with that obtained from the normal serum illustrated in Fig. 6.

A COMPARISON OF THE SPECTRA OF THE CHROMATIC GROUPS OF DIFFERENT HEMOCYANINS

It is a question of considerable interest to what extent the various respiratory proteins may be regarded as distinct "inventions of Nature," especially in that it is desirable to know whether the possession

of similar or identical respiratory pigments indicates a generic relation between the groups of organisms possessing them. Recently much evidence has accumulated establishing the fact that the various hemocyanins are specifically different substances. This evidence consists in the demonstration of distinctive differences in the physical and chemical properties of these proteins. On the other hand, the evidence regarding the ratio between oxygen-combining power and copper content of the hemocyanins indicates that these substances have certain points in common, at least with regard to the portion of the molecule concerned with

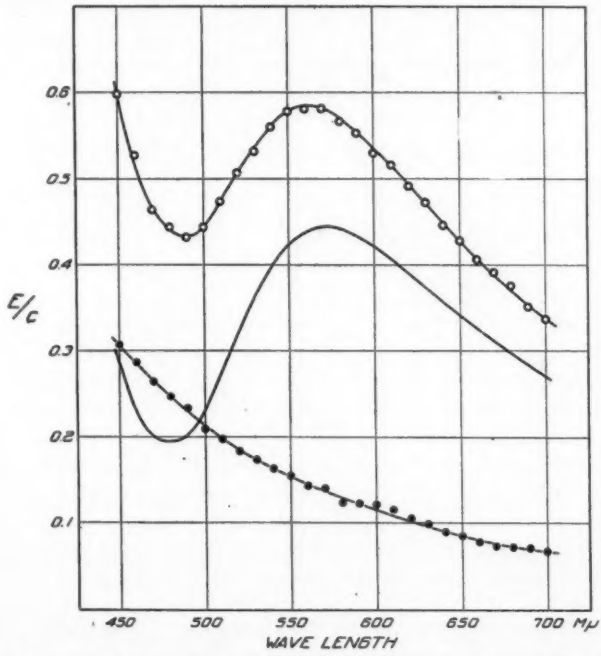

FIG. 9. Absorption spectrum of blood of *Homarus americanus* after extracting the pigment with chloroform. Upper curve, oxygenated blood; lower curve, reduced blood; intermediate curve, the spectrum of the chromatic group. Copper content of blood 0.0522 mgm. per cc.; dilution, 2 parts of blood plus one part of solution containing 0.4 mols NaCl, 0.01 mols KCl, 0.02 mols $CaCl_2$ per liter; pH 8.05; length of absorption vessel 3.3 cm.

this function. To this complex when combined with oxygen we have applied the designation "chromatic group." A comparison of the spectra of the chromatic groups of different forms should consequently give evidence regarding the similarity of the chromatic groups in the

hemocyanins of different classes of animals. In Fig. 10 the corrected spectra of the chromatic groups of the four species which we have studied are collected. It may be seen that on the whole the curves are strikingly alike, not only with regard to their shape, but also in relation to the actual quantity of light absorbed by equal numbers of chromatic

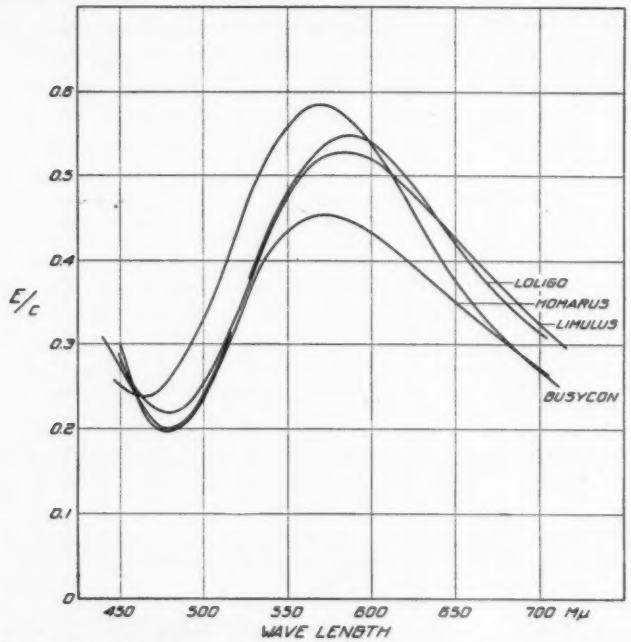

FIG. 10. Absorption spectra of chromatic groups of blood of *Busycon*, *Limulus*, *Loligo* and *Homarus*. For data see Figs. 3, 4, 5 and 6.

groups. One is forced to the conclusion that the complexes responsible for these spectra are very much alike in each case. On the other hand, there are unquestionable differences between the spectra in the different cases.

FACTORS AFFECTING THE ABSORPTION OF LIGHT BY THE CHROMATIC GROUP

A comparison of the chromatic groups of different species raises the question as to whether the differences observed may be attributed to differences in the chemical make-up of the body fluids in question.

It is consequently desirable to examine the effect of the nature of the solvent upon the absorption of light by hemocyanin solutions.

Hydrogen Ion Concentration.—The first point to be considered is the influence of hydrogen ion concentration upon absorption and scattering. When specimens of *Limulus* blood, to which various amounts

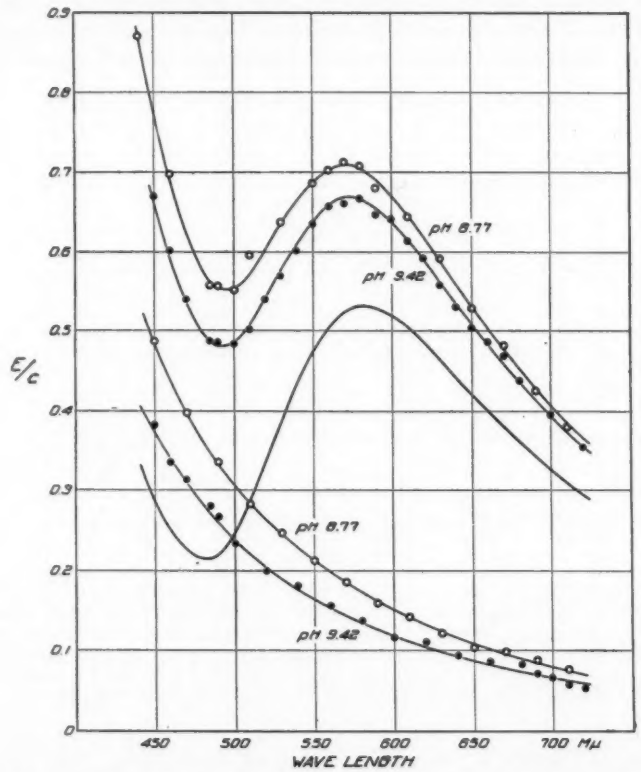

FIG. 11. Absorption spectra of blood of *Limulus polyphemus* at different hydrogen ion concentrations. Upper curves, oxygenated blood at pH 8.77 (hollow circles) and pH 9.42 (dots); lower curves, the same after reduction; intermediate curve, the spectrum of chromatic group, which is identical in both cases. Copper content 0.081 mgm. per cc.; length of absorption vessel 3.3 cm. Dilution which gave pH 8.77: 20 parts blood, 35 parts sea water, 5 parts 0.04N NaOH; dilution which gave pH 9.42: 20 parts blood, 35 parts sea water, 5 parts 0.1N NaOH.

of acid or alkali have been added, are examined, it is obvious to the eye that the color of the solution more alkaline than about pH 9 is different from the others. This difference is evident not only in the

oxygenated, but also in the reduced solutions, the oxygenated solution being a purer blue beyond pH 9 and the reduced solution having a fainter yellow color. In Fig. 11 are illustrated absorption spectra of specimens of oxygenated and reduced *Limulus* blood which were diluted with sea water, to which small quantities of sodium hydroxide had been added so that the solutions were at pH 8.77 and 9.44 respectively. With these curves the data presented in Fig. 4 should be compared, as the latter was obtained from the same blood brought to pH 6.05

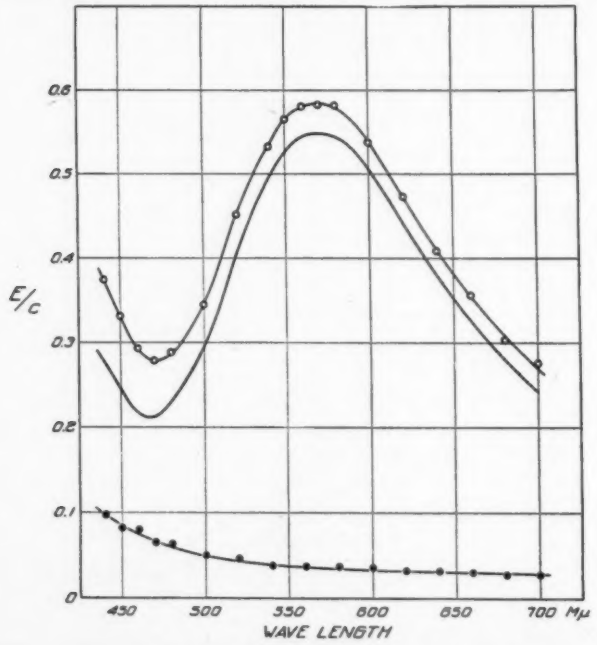

FIG. 12. Spectra of hemocyanin of *Busycon canaliculatum*. Upper curve, oxygenated; lower curve, reduced; intermediate curve, spectrum of the chromatic group. Hemocyanin purified by precipitating four times with saturated ammonium sulfate followed by dialysis. It contained 0.129 grams hemocyanin per cc. and 0.308 mgm. Cu per cc. Dilution, 2 parts hemocyanin solution plus 14 parts H_2O plus one part 0.1N NaOH; pH 9.16; length of absorption vessel 3.3 cm.

by the addition of sea water containing small quantities of hydrochloric acid. The spectra illustrated in Figs. 4 and 11 account for the observed differences in color. The more alkaline specimen absorbs less light than the others in both the oxygenated and the reduced conditions.

It is clear also that the more alkaline solution scatters less light than do the others. Comparison of the corrected spectra of the chromatic groups shows, on the other hand, that the true absorption of light is not changed to a detectable degree by alterations in the hydrogen ion concentration. The differences in the spectra of the oxygenated bloods are sufficiently accounted for by the differences in scattering.

Salts.—A more profound alteration in the solvent may be obtained by purifying the hemocyanin so that it may be dissolved in water

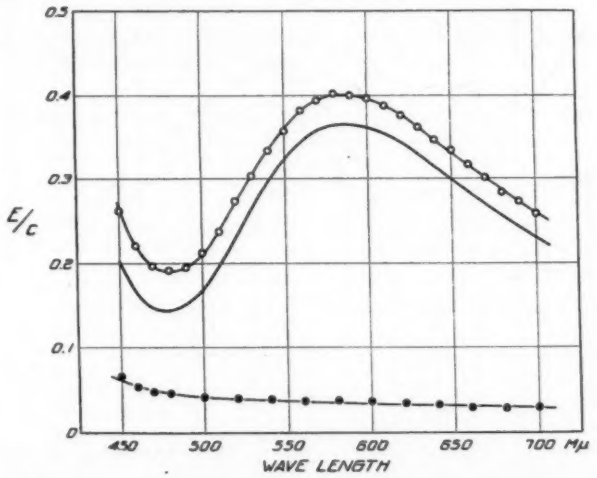

FIG. 13. Spectra of hemocyanin of *Limulus polyphemus*. Upper curve, oxygenated; lower curve, reduced; intermediate curve, spectrum of the chromatic group. Hemocyanin purified by precipitating four times with saturated ammonium sulphate followed by dialysis. It contained 0.109 grams hemocyanin per cc. and 0.184 mgm. Cu per cc. Dilution, 5 parts hemocyanin solution plus 12.5 parts H_2O plus 2.5 parts 0.1N NaOH; pH 9.10; length of absorption vessel 3.3 cm.

practically free of salts or other substances. By this means it is possible to compare the spectra of the chromatic groups of the different hemocyanins in solutions which are more or less identical. When solutions of pure hemocyanin are compared, it may be observed that the Tyndall phenomenon has undergone great diminution. Dilute solutions of reduced hemocyanin are practically colorless. The oxygenated solutions are of a purer blue color than when these substances are dissolved in the blood. These characteristics are all accounted for by an examination of the absorption spectra of the solutions, in which it may be observed that the reduced solutions appear to absorb very little light

and to absorb only slightly more light at the violet end of the spectrum than at the red end. Similarly the transmission of light in the blue-green region of the spectrum of the purified oxygenated hemocyanin is much greater than in the case of blood, and the absorption spectrum of the oxygenated solutions does not differ greatly from those of the corrected spectra of the chromatic groups. Spectra of purified hemocyanin solutions of *Busycon, Limulus* and *Homarus* are illustrated in

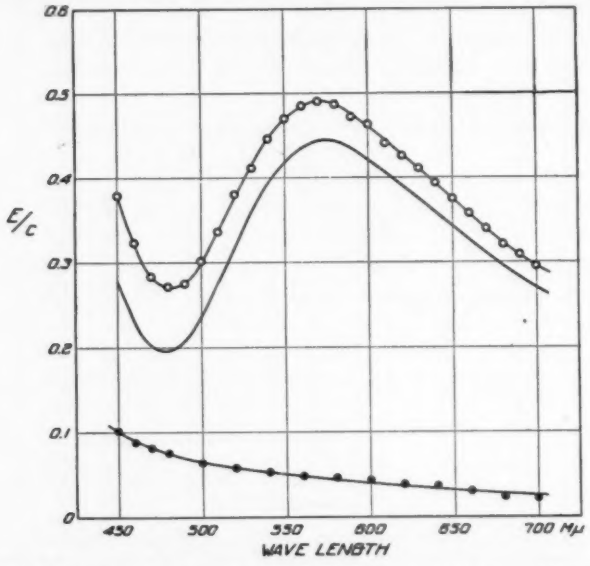

FIG. 14. Spectra of hemocyanin of *Homarus americanus*. Upper curve, oxygenated; lower curve, reduced; intermediate curve, spectrum of the chromatic group. Hemocyanin, purified by dialysis. Solution contained 0.1185 grams dry solids per cc. and 0.196 mgm. Cu per cc. Dilution: one part hemocyanin solution plus 3 parts H_2O; pH 8.10; length of absorption vessel 3.3 cm.

Figs. 12, 13 and 14, together with the corrected spectra of the chromatic groups.

Comparison may now be made between the spectra of the chromatic groups of the purified hemocyanin and of the native blood. This is done in the case of these three species in Tables I, II and III. For accurate comparison the value of E/c for each wave length in the case of the purified hemocyanin is compared with its value in the case of the native blood. If the spectra of the chromatic groups are identical, this ratio should be the same at all wave lengths and have the value 1.0.

Examination of the tables showed that the ratio is not quite constant in each case at different wave lengths. The divergences are not large, but appear to be reproducible and indicate that the spectrum of the chromatic groups undergoes certain small changes as the result of the process of purification. The ratio also deviates from the value of 1.0 in each case. With *Busycon* and *Homarus* the purified material absorbs only slightly less light at each wave length than does a like concentration

TABLE I

Comparison of absorption of light by chromatic groups of blood and purified hemocyanin of Busycon canaliculatum.

Wave Length	Hemocyanin	Blood	Ratio
$m\mu$	E/c	E/c	
460	0.216	0.238	0.908
480	0.230	0.260	0.885
500	0.296	0.333	0.890
520	0.406	0.436	0.932
540	0.497	0.530	0.938
560	0.546	0.580	0.942
580	0.546	0.575	0.950
600	0.506	0.537	0.942
620	0.442	0.473	0.935
640	0.380	0.403	0.942
660	0.325	0.348	0.935
680	0.280	0.304	0.923
700	0.242	0.268	0.904

of hemocyanin in native blood. In the case of *Limulus* the discrepancy is much greater, amounting to about 30 per cent. These differences might be due to an alteration in the absorption of light by each chromatic group. On the other hand, they might be adequately accounted for on the assumption that as the result of the process of purification a certain quantity of the hemocyanin has lost the ability to combine with oxygen, which is necessary in order that the chromatic group be formed. The difference in the case of *Limulus* is sufficiently large to allow this possibility to be tested by a determination of the oxygen-combining power of the solution. The hemocyanin solution employed in this case contained 1.93 milligram atoms of copper per liter and might be expected to have an oxygen capacity of 1.93 milligram atoms of oxygen per liter. Actual analyses of the oxygen content of this solution when equilibrated with air yielded the values, 1.94, 1.95, 1.90 (mean 1.93) milligram atoms of oxygen per liter. Allowing 0.50 milligram atoms of oxygen per liter dissolved in the solution, one obtains 1.43 milligram

atoms as the actual oxygen-combining capacity. This value is 74 per cent of the theoretical, indicating that 26 per cent of the hemocyanin had lost its ability to combine with oxygen. The absorption of light by this solution is approximately 70 per cent of the absorption to be expected from the observations on hemocyanin as it occurs in native blood as Table II shows. It seems clear that in the case of this specimen at least, the discrepancy between the spectrum of blood and of the purified hemocyanin solution is due in large part to the modification of a portion of the hemocyanin in the process of preparation or preservation. The hemocyanin from which this specimen was prepared had been preserved for many months precipitated in half-saturated ammonium sulfate prior to preparation, and unfortunately we have not had an opportunity of re-examining this question with freshly collected hemocyanin.

TABLE II

Comparison of absorption of light by chromatic groups of hemocyanin and blood of Limulus polyphemus.

Wave Length	Hemocyanin	Blood	Ratio
mμ	E/c	E/c	
460	0.165	0.244	0.677
480	0.144	0.220	0.655
500	0.168	0.257	0.655
520	0.231	0.338	0.683
540	0.298	0.438	0.680
560	0.345	0.506	0.683
580	0.365	0.528	0.692
600	0.362	0.518	0.699
620	0.344	0.486	0.708
640	0.314	0.446	0.705
660	0.284	0.403	0.705
680	0.256	0.362	0.708
700	0.231	0.324	0.714

These results lead to the conclusion that the observed differences in the extinction coefficients of hemocyanin in blood and in purified solutions may be accounted for largely by the denaturation of the hemocyanin in the process of preparation. They do not demonstrate that some difference in the absorption of light by the chromatic groups does not occur. Unfortunately the precision of the available methods for measuring oxygen capacity in these solutions is so low that changes cannot be detected unless they are relatively large. It may be concluded, however, that the spectra of the chromatic groups vary very

little as the result of freeing the solutions from electrolytes and other impurities.

A comparison of the absorption of light by the reduced solutions of purified hemocyanin illustrated in Figs. 12, 13 and 14, with the curves for the absorption of light by the reduced serum of the corresponding species, shows that in the purified preparations, the scattering of light is much less than in the native blood. In the case of the lobster, the values of E/c characteristic of each wave length are, in the

TABLE III

Comparison of absorption of light by chromatic groups of hemocyanin and blood of Homarus americanus.

Wave Length	Hemocyanin	Blood	Ratio
$m\mu$	E/c	E/c	
460	0.232	0.240	0.968
480	0.196	0.202	0.972
500	0.237	0.244	0.972
520	0.317	0.332	0.956
540	0.393	0.410	0.959
560	0.435	0.447	0.975
580	0.444	0.451	0.984
600	0.421	0.432	0.976
620	0.391	0.402	0.973
640	0.358	0.366	0.979
660	0.327	0.333	0.984
680	0.295	0.300	0.984
700	0.270	0.268	1.007

purified serum, about one-half those characteristic of the reduced blood. In the blood of *Busycon* and *Limulus,* the scattering of light is many times greater than in the purified preparations.

The effect of purification upon the scattering of light may be shown to be due primarily to the removal of electrolytes from the solvent of the hemocyanin. By adding salt to purified hemocyanin solutions, the scattering effect is greatly increased. At the same time, the spectrum of the oxygenated solution approaches more nearly that of native blood. The spectrum of the chromatic group, however, appears to remain unchanged. These facts are illustrated by the data in Table IV, in which the values of E/c for oxygenated and reduced solutions of *Busycon* hemocyanin are compared when it is dissolved in water and when it is dissolved in a solution of potassium phosphate of an ionic strength approximately equal to that of native blood.

It may be concluded from the foregoing that the spectrum of the chromatic group is a relatively constant characteristic of hemocyanin solutions, influenced little if at all by the composition of the solvent provided that this does not interfere with the oxygenation of the material. On the other hand, the apparent absorption of light due to scattering varies greatly with the nature of the solvent and particularly with its salt content and hydrogen ion concentration. These facts are essential to the use of photometric methods in examining these solutions. They demonstrate that the measure of the absorption of light by the

TABLE IV

Absorption of light by hemocyanin of Busycon canaliculatum dissolved in potassium phosphate buffer; phosphate concentration, 0.357 molar; pH, 7.7.

Wave Length	Oxygenated in Phosphate	Reduced in Phosphate	Chromatic Group in Phosphate	Chromatic Group—Salt-Free	Ratio
mμ	E/c	E/c	E/c	E/c	
460	0.559	0.345	0.214	0.216	1.009
480	0.514	0.288	0.226	0.230	1.017
500	0.544	0.237	0.307	0.296	0.964
520	0.625	0.208	0.417	0.406	0.974
540	0.691	0.178	0.513	0.497	0.969
560	0.722	0.131	0.567	0.546	0.963
580	0.695	0.132	0.563	0.546	0.971
600	0.632	0.118	0.514	0.506	0.985
620	0.560	0.105	0.455	0.442	0.972
640	0.477	0.092	0.385	0.380	0.987
660	0.415	0.078	0.337	0.325	0.965
680	0.361	0.071	0.290	0.280	0.966
700	0.324	0.069	0.255	0.242	0.950

chromatic group may be a reliable index of the concentration of oxyhemocyanin. They also make it clear that in such measurements every precaution must be taken to control and take account of the degree of absorption due to the scattering of light.

In a preliminary report on the present investigation (Redfield, 1929) it was suggested that the relative size of the particles of hemocyanin could be deduced from the scattering of light with the aid of the Rayleigh theory. However, Raman (1927) has developed a theory of scattering by colloidal solutions, in accordance with which it appears possible to relate the observed optical phenomena to the osmotic pressure of the solutions. The experiments of Loeb on gelatin indicate that the variations in osmotic pressure of protein solutions induced by altering the nature of the solvent, which he accounted for by the

considerations involved in Donnan membrane equilibria, are in the necessary direction and have sufficient magnitude to account for the observed variations of scattering in terms of Raman's theory. Until this possibility is examined critically, it is improper to draw inferences

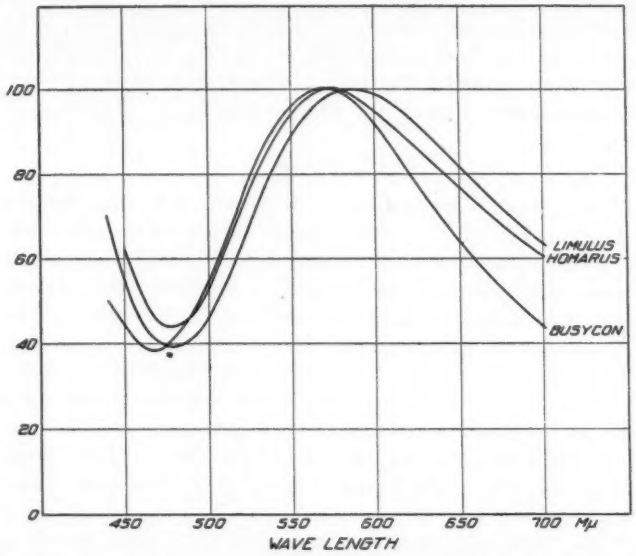

FIG. 15. Absorption spectra of chromatic groups of purified hemocyanins of *Busycon*, *Limulus*, and *Homarus*. The ordinate is an arbitrary scale such that the value of E/c for each spectrum is 100 at the wave length of maximal absorption. For data see Figs. 12, 13 and 14.

concerning the degree of aggregation of hemocyanin in blood from the phenomenon of scattering.

COMPARISON OF THE CHROMATIC GROUPS OF PURIFIED HEMOCYANIN IN AQUEOUS SOLUTIONS

In order to compare the spectra of the chromatic groups of the different purified hemocyanins it is necessary to employ some method which disregards the errors due to the denaturation of a certain portion of the hemocyanin in the process of purification, as the foregoing discussion indicates that data may not give us accurate information with regard to the concentrations of oxygenated hemocyanin in the various preparations. The spectra of the chromatic groups of the different hemocyanins described by Figs. 12, 13 and 14 have consequently been reduced to an arbitrary scale in which the maximal in-

tensity of absorption in the yellow region has been taken as 100. The data so obtained are plotted in Fig. 15. Comparing these curves, it is evident that even in aqueous solutions the spectra of the chromatic groups are markedly different. One may conclude consequently that the characteristics of these spectra are not dependent upon the chemical peculiarities of the body fluids of the different animals but on specific differences in the chromatic groups themselves or on the influence of the specific characteristics of the hemocyanin molecule as a whole upon that portion which is concerned with the transport of oxygen.

Summary

1. The absorption of light by the blood and by purified preparations of the hemocyanin of the conch, *Busycon canaliculatum*, the horse-shoe crab, *Limulus polyphemus*, the squid, *Loligo pealei*, and the lobster, *Homarus americanus*, has been studied. It is shown that the absorption of light by solutions containing oxygenated hemocyanin may be resolved into two components: (a) that due to the true absorption by the chromatic group formed by the union of oxygen with the portion of the molecule containing copper and (b) that due to the scattering of light by the dissolved protein.

2. In the analysis of the spectrum of the blood of the lobster, the absorption of light by the pigment tetronerythrin has been taken into account.

3. The spectrum of the chromatic group of a given species varies very little, if at all, as the result of alterations in the hydrogen ion concentration and salt content of the solution.

4. The spectra of the chromatic groups of the different species display a considerable similarity, indicating a close chemical relationship. There exist, however, definite differences in the spectra of each species which persist after the process of purification and indicate definite specific differences in the various hemocyanins.

5. The scattering of light varies widely among the different species and is responsible in large part for the difference in appearance of the bloods, particularly when viewed by reflected light. The scattering is modified greatly by changes in the composition of the solution, being diminished in the more alkaline solutions and particularly in solutions free from electrolytes.

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ANOPLOPHRYA MARYLANDENSIS N.SP., A CILIATE FROM THE INTESTINE OF EARTHWORMS OF THE FAMILY LUMBRICIDÆ

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Material and Methods

Anoplophrya marylandensis, a new species of astomatous ciliate, was discovered in the intestine of *Lumbricus terrestris* (Linn., 1758) and *Helodrilus caliginosus* (Savigny, 1826). Many of the infected hosts were immature forms, making identification uncertain. Assistance in identifying the earthworms given by Dr. Frank Smith, formerly of the University of Illinois, is gratefully acknowledged.

The hosts infected with this form were obtained from a limited area in the city of Baltimore. Hosts of the same species obtained from three other localities within the city failed to show this form. Those from two of the other regions showed no intestinal ciliates. In the infected area the incidence of infection was 29.13 per cent.

The parasites were usually numerous in the infected worms. They were found only in the anterior third of the intestine, and were most numerous just in back of the gizzard. The organisms were obtained after anesthetizing the host with chloretone. The body of the worm was slit along the mid-dorsal line exposing the intestine into which short incisions were made in different regions. The contents of the intestine were removed from these regions with a tooth-pick and smears were made in physiological salt solution. Smears were fixed with Schaudinn's fixative and were stained with Heidenhain's iron hæmatoxylin. Parasites were studied in the living condition by placing the contents of the intestine into a watch glass of physiological salt solution.

Description

This new species was uniformly ciliated and flattened. The body was extremely thin and did not appear to be more than one-fifteenth its width in thickness. Stained specimens were not found in such a position that thickness could be measured. The dorsal and ventral surfaces of specimens just removed from the intestine appeared to be perfectly flat. Some of them became slightly rounded after they had

been in the physiological salt solution for a few minutes. The change in form was evidently due to a difference in osmotic pressure. The body was rounded at the posterior end and slightly pointed at the anterior end; it was broader at the anterior end than at the posterior. The greatest breadth was just anterior to the center of the body.

One hundred specimens were measured with an ocular micrometer at a magnification of 1000. The following dimensions and biometrical data were entered as being typical.

Breadth in microns	Length in microns										
	36	40	44	48	52	56	60	64	68	72	
16		1	1								2
20	1	5		3							9
24	1	2	5	5	9	1	2	1			26
28		1	7	2	1	2	1	3	2		19
32	1		1	2	7	8	5	3			27
36					1	4	5	3			13
40								1	1	2	4
	3	9	14	12	18	15	13	11	3	2	100

	Length	*Breadth*
Range	36 to 72 μ	16 to 42 μ
Mean	52.7 $\pm$ 0.6 μ	28.6 $\pm$ 0.4 μ
Standard Deviation	8.6 $\pm$ 0.4 μ	5.6 $\pm$ 0.3 μ
Coefficient of Variation	16.4 $\pm$ 1.0%	19.8 $\pm$ 1.0%

The body was covered with long cilia arranged in longitudinal rows which were close together. The number of rows varied from 31 to 40 in five specimens. These longitudinal rows of cilia converged slightly at each end of the parasite. The cilia of twelve specimens averaged 7.3 μ in length. The average length of these twelve parasites was 50.8 μ. In general the length of the cilia varied directly with the size of the parasite, the larger parasites having the longer cilia.

There was a thin pellicle which covered a transparent layer of ectosarc. The ectosarc was confined to a thin layer except at the anterior end where it made up the greater part of the pointed region. The endosarc was very granular when stained with Heidenhain's iron

hæmotoxylin and it appeared to have many chromatin granules scattered throughout.

There were two nuclei, a large ribbon-like macronucleus and a small spherical micronucleus. The macronucleus extended through the long axis of the body and was nearly as long as the body itself. In 25 specimens the average length of the macronucleus was 43.1 μ while the average length of these same parasites was 55.5 μ. The average width of the macronucleus was 5.9 μ while the average width of the parasites was 32.7 μ. The outline of the macronucleus was very irregular and several showed fine projections extending into the endosarc. (Fig. D.) Often a clear space appeared around the macronucleus in stained specimens. This was probably due to shrinkage.

The micronucleus was very small and appeared to be spherical in specimens not undergoing division. It was surrounded by a layer of clear protoplasm, which may also have been due to shrinkage, for the micronucleus could only be seen in stained specimens. The micronucleus was found about mid-way between the anterior and posterior ends of the body and was lateral to the macronucleus. It was always found on the side of macronucleus opposite to that of the row of contractile vacuoles.

If the parasites were numerous in the host, about every tenth one was in the act of dividing. Division was transverse. Figs. B, C, D, and E show different stages in transverse fission. The micronucleus underwent a mitotic division with the formation of a spindle and chromosomes. There were apparently only a few chromosomes but their extremely small size made them very difficult to count. Fig. C shows four chromosomes that have just divided and the two groups are separating from each other. In the prophase of division the chromatin granules appeared to be lined up in two strings with successive enlargements which made them look like two strings of beads.

Tarnogradsky (1914) and Cépède (1909) have described posterior budding in other species, but in the present study no specimens in unequal division have been found nor did any have other individuals attached to them.

No conjugating individuals were seen as has been described by Collin (1909) in *A. brasili* Leger and Duboscq.

There was a single longitudinal row of contractile vacuoles along one side of the macronucleus. The number varied from two to five. The vacuoles in even a single specimen were of widely different sizes. As many as five different specimens have been watched for one half hour at a time and never have any of the vacuoles been seen to contract. The specimens have been placed in a suspension of India ink but no

expulsion of fluid from the vesicles was seen. Lankester (1870) described the contraction of vacuoles in an Opalina which has since been classified as *Anaplophrya naidos* by Kent (1880) and more recently as *Bütschliella naidos* by Mackinnon and Adam (1924). Lankester said the contractile vacuoles in this form contracted very suddenly, slowly reappearing in the same place. He found that the collapse occurred a little less frequently than twice a minute. Tarnagradsky (1914) found that the period of contraction of the contractile vacuoles in *A. inermis* Stein was from 1.5 to 15 minutes. Cépède (1910) found that the contractile vacuoles of *A. alluri* failed to contract if the animals were removed from their normal habitat.

Behavior

Parasites could not be kept alive more than twenty-four hours outside the host. Physiological salt solution and various dilutions of it with distilled water to 0.25, 0.5, 0.375, 0.625, and 0.75 of its normal strength were used. The parasites lived longest in the normal solution. Those in the less concentrated solutions soon developed large blisters on the body and died.

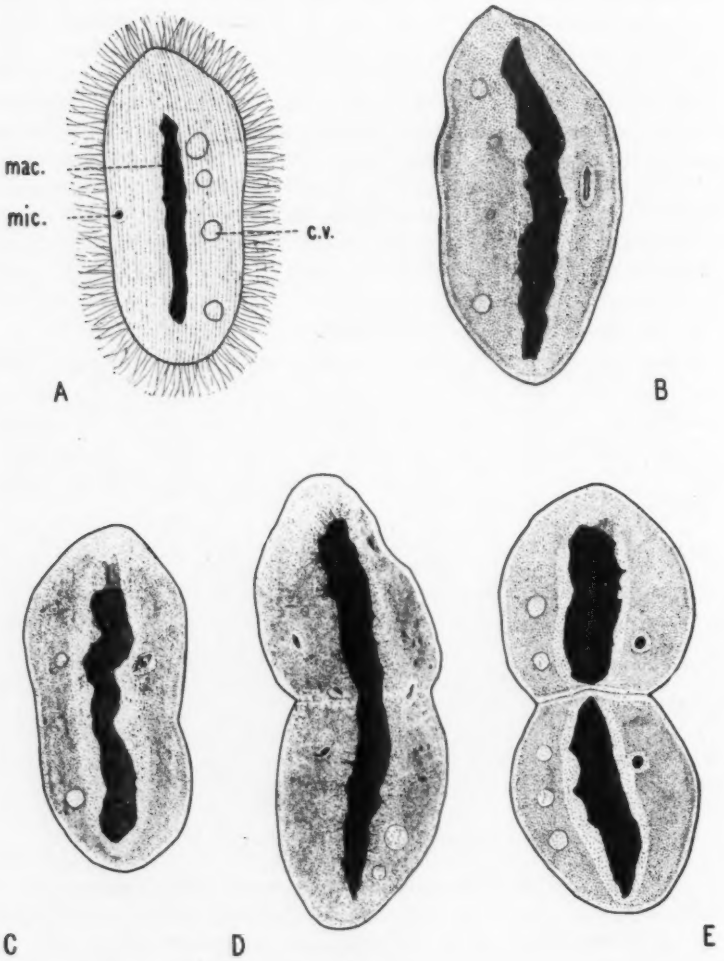
The parasite turned on its long axis as it swam, frequently making complete turns but often making only a half turn from a horizontal position to a horizontal position and back again. It was observed to make this half turn even when the liquid in which it was confined was sufficiently deep so that the animal was not cramped nor was it prevented from making a complete turn. When it did make a complete turn it was in a clockwise direction.

The parasite swam rapidly and underwent no deformation due to the mechanical action of movement, though the body seemed flexible enough to bend when it struck an obstruction.

Differential Diagnosis

As far as available literature goes to show, *Anoplophrya marylandensis* n.sp.,[1] can with one exception be distinguished from all other species in the genus which fall within its size range (36 to 72 μ in length) by the fact that it has one row of contractile vacuoles instead of two rows. The exception is *A. parva* Rossolimo, from an aquatic oligochæte and is separated from *A. marylandensis* by the position of the micronucleus. In *A. marylandensis*, the micronucleus occurs upon the side of the macronucleus opposite the contractile vacuoles; in *A. parva*, both micronucleus and contractile vacuoles are on the same side

[1] A type specimen of this species has been deposited in the National Museum at Washington, D. C.



EXPLANATION OF FIGURES

All specimens stained with Heidenhain's iron hæmotoxylin. All drawings made with camera lucida at a magnification of × 750.

FIG. *A*. *Anoplophrya marylandensis* n.sp., normal resting individual. Mac., macronucleus; mic., micronucleus; c.v., contractile vacuole.

FIGS. *B*, *C*, *D*, *E* represent successive stages in division of *A. marylandensis*. *B*, prophase, *C*, metaphase, *D* and *E*, telophase.

of the macronucleus. Furthermore, *A. marylandensis* is much broader (averaging about 28 μ in breadth) in comparison to *A. parva* (breadth, 17 μ).

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THE GROWTH OF LARVÆ OF *AMBYSTOMA MACULATUM* UNDER NATURAL CONDITIONS

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Introduction

Attempts to describe the increment in length and weight during the larval history of amphibians are confronted with either of two difficulties. If the animals are raised in the laboratory at a constant temperature, the normal or optimum food conditions cannot be duplicated readily; if animals are collected periodically from their natural habitat, the conditions of life there are so variable as to produce data difficult to describe. Accordingly the literature is neither extensive nor consistent.

Davenport (1897, 1899) presented a few data upon the weight increment of larvæ of "the common frog" under laboratory conditions. Three stages of growth are recognized; first a period of slow growth accompanied by abundant cell division, then a period of rapid growth due to imbibed water, and finally a period of equally rapid growth in which the increment is due to increase both in organic substance and water.

Schaper (1902) has provided data still more complete on the larval growth of *Rana esculenta* under laboratory conditions. The daily growth in weight and volume of these tadpoles is slight at first, gradually becoming greater and attaining a maximum value at about eighty days. During the following week these values fall to about half as the animal undergoes metamorphosis. During the first fourteen days of development the organic matter and ash remain constant (13 mgm. and 1 mgm. respectively). Weight increment during this period is due solely to imbibition of water. The percentage of solid then increases until maximum size is attained; during metamorphosis this percentage is further increased. Schaper's data on length increase cannot be easily interpreted, probably because too few specimens were considered.

Robertson (1923) apparently unaware of Schaper's work has attempted with indifferent success to convert Davenport's data into a mathematical expression. He believed that the weight increment in the frog tadpole could be expressed by a single symmetrical sigmoid curve.

Studies on salamanders have shown variable rates of growth. When the length-age data of Eycleshymer and Wilson (1910) on *Necturus* under laboratory conditions and those of Bishop (1926) dealing with the animal under natural conditions are plotted it may be seen that during the first three (or four) months of development (except for a period before the embryonic axis is straight) the length increases at a constant rate.[1] The nearly uniform yearly growth to the period of sexual maturity, which Bishop records, suggests that aside from thermal variations the rate of linear growth may be constant from year to year.

Wilder (1924), on the other hand, has shown that under natural conditions the rate of linear growth varies at different times during the larval history in *Spelerpes bislineata*. Although she disregarded the embryonic development, subsequent growth stages are recorded. The post-embryonic stage, until the yolk is absorbed, is the period of most rapid growth. The typical larval period during the fall and winter of the first year is a time of slow growth. The latter part of this period during the spring of the second year is characterized by rapid growth. The premetamorphic stage during the fall and winter of the second year involves a period of slow growth, then a period of fluctuating growth. The metamorphic stage in the summer of the third year is a period in which the catabolic changes are more pronounced than the anabolic.

It must be noted that *Spelerpes* does not become terrestrial until it has spent two years of its life in an aquatic habitat; *Necturus* is permanently aquatic. It seems likely that a more typical method of growth would be found in salamanders which have an aquatic stage lasting for only a single season. The increase in length of *Ambystoma* has been studied by two observers. Uhlenhuth (1919), who has studied *A. opacum* under constant conditions, stated that the rate of growth seems to be proportional to the velocity of metamorphosis (rate of growth $\times$ age at metamorphosis $=$ constant). He does not, however, describe the growth rate of various periods of development.

Patch (1927) has described the length increase in three groups of *Ambystoma* as consisting of two sigmoid curves, one embryonic and the other larval. The point of junction of these two curves is 15.61 mm. in *A. maculatum*, 14.07 mm. in *A. tigrinum*, and 11.96 mm. in the axylotl.

In order for curves of length increase to represent a fundamental

[1] Actually, Bishop's data shows a slight variation from the constant growth rate over a period of two weeks in July. The climatological records of the U. S. Weather Bureau for the Saegertown, Pa. region give a rise in temperature during this period which undoubtedly accounts for the fluctuation.

phase of growth, the relation between weight and length for successive stages must be constant, as Miss Patch has assumed. In view, however, of the marked changes in body form during the embryonic life of *Ambystoma* it seems unlikely that the "indices of build" are uniform. It is necessary to have data on both length and weight, at least to the point in development where the body form assumes nearly constant larval proportions, in order to correctly appreciate the body increment.

Material and Methods

During the spring of 1928, the author located a salamander pond sufficiently well populated with spawning *Ambystoma maculatum* to indicate that eggs and larval specimens could be obtained throughout the season. The present study involving about 1700 specimens is the outcome of two years of systematic collection from that habitat.

Delhi Pond is a shallow, sheltered, leafy-bottomed forest pond in the environs of Ann Arbor. It has a maximum area of one-twelfth acre and a maximum depth of about four feet; it is ordinarily a permanent pond but became dry during the season of 1929. In addition to *A. maculatum*, the usual invertebrate fauna, *A. tigrinum* and *Rana cantabrigensis* were present.[2]

From the time when eggs were first observed until the salamanders metamorphosed, periodic random collections were made, the intervals between successive collections being seldom more than a week. Thirty to forty specimens formed a sample although occasional collections, particularly during the early stages, amounted to more than a hundred specimens. Specimens were collected by dredging the bottom of the pond with a hand net formed of a yard of wire netting stretched between two poles. All parts of the pond were sampled so that the collection is quite representative. The maximum-minimum temperatures of the pond were recorded at the time of collection.

The specimens were brought to the laboratory alive, anesthetized and measured, mutilated specimens having been discarded. During the non-motile stages anesthesia was not required. The eggs and embryos up to the period of hatching were placed for measurement upon the stage of a binocular microscope fitted with a camera lucida.

[2] Although B. G. Smith (1911) stated that *Ambystoma tigrinum* and *A. maculatum* are not found in the same habitat in the Ann Arbor region, the author found both salamanders in abundance in the present location. The two populations are more or less independent of one another and it is not evident that the larger salamander through its predatory activities seriously affects the *A. maculatum* population. The eggs and larvæ of the two species could not be confused easily since the egg clutches, the time of hatching, the size and appearance of embryos and larvæ are distinctly different.

The image of the embryo could thus be superimposed on a properly calibrated scale. The larger, later specimens were measured by means of drafting calipers and millimeter rule. In the blastula and non-motile stages the maximum diameter or length irrespective of the curvature of the body axis in relation to the yolk mass was recorded. The length taken in later stages when the axis was linear was the maximum length.

The average weight at different developmental stages was also determined: The anesthetized salamanders were placed in a tared crucible and weighed after the excess water had been absorbed by pipette and filter paper. They were then dehydrated for several days in a drying oven at 95–97° C. and the dry weight determined. Following this the sample was incinerated over a Meeker burner for two to ten hours and the ash weighed.

Results

Changes in Weight to the Period of Metamorphosis.—During the year 1928, the first eggs were found on April 3. On August 18, many specimens had begun to metamorphose at a weight of about 1200 mgm. A week later there were relatively few specimens in the pond. During the year 1929 the first eggs were collected on March 27, and on August 14, a number of specimens, at approximately 800 mgm. had metamorphosed. When the average weight of a sample of salamander eggs, embryos or larvæ is plotted against the age, as in Figs. 1 and 2, the rate of growth may be expressed as a curve. The weight increment was slow at first, gradually increasing to the middle of June when the rate of increase became more and more rapid. By the middle of July the rate of growth was at its maximum. In the first week of August the growth rate was markedly reduced. Finally growth became negligible and metamorphosis occurred. The weight increase may be thus described as a single sigmoid curve. Under natural conditions the first stages of growth were considerably prolonged, due to the low water temperatures of spring. During the larval and premetamorphic stages the temperatures are more nearly the same. The terminal period of growth is very brief, so short in fact, that the curve of *Ambystoma* increment shows a marked variation from the curves of autocatalytic growth in other animals. During both years the same general type of sigmoid curve is demonstrated, although the actual weight values are considerably different.

Linear Increase to the Time of Metamorphosis.—The curve formed by plotting length against age shows a slight deviation during the first four or five weeks of development. From this point on the curve is sigmoid. Growth increment is gradually increased to a period within

three to four weeks of metamorphosis when the rate of increase is considerably lowered, and finally becomes negligible. The deviation during the early stages of development, which Miss Patch has interpreted as a distinct period of growth, involving the typical sigmoid growth rates, is due to the form changes of the embryo. The embryonic axis from the neurula to the early limb bud stages is curved around the

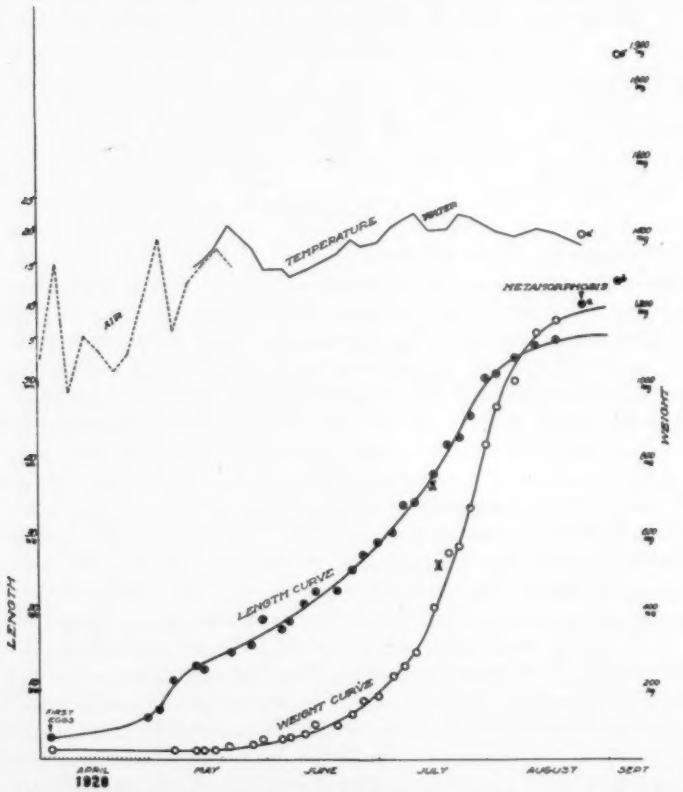

FIG. 1. A curve showing the relation between average length and weight of larval specimens of *A. maculatum* at various ages. Data of 1928.

yolk mass. Increase in length of this axis during the early growth stages does not result in equivalent increase in the total length of the embryo. It is not until the embryonic axis becomes straightened that the total length shows marked increase.

That the departure from a single sigmoid curve during the early development is not a distinct phase of growth may be demonstrated. When an "index of build" (Length ^{3}/Weight) is computed (Table I), it is clearly indicated that length and weight are not directly associated during the early stages. This index varies constantly to a period shortly before hatching when the embryonic axis becomes linear. It is fairly constant, however, for the free living larval stages.

A group of experiments carried under approximately constant temperature conditions gives indication that the degree of curvature may vary under environmental circumstances. Four groups of salamanders

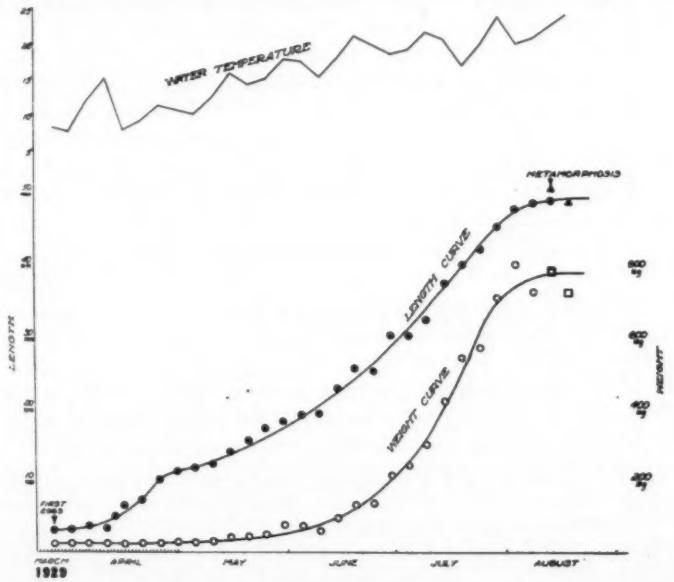

FIG. 2. Curve of growth showing weight and linear increment. Data of 1929.

at the neurula stage were placed at approximately constant temperatures of 4° C. ± 1, 13° C. ± 1, 19° C. ± 1 and 27° C. ± 1. When the four groups of length-age data acquired from these animals were plotted in such a way as to rule out the change in growth rate due to temperature, that is, when all the data, after allowance is made for appropriate thermal coefficients of growth, are plotted as though the animals were raised at 19° C., the shape of the curve is not the same for each group. The linear increase in the 4° sample was practically

TABLE I

Giving the average lengths, weights and analyses at different stages of embryo and larval A. maculatum. Data of 1929

DATE	MEAN LENGTH	AVERAGE WET WEIGHT	LENGTH³/WEIGHT	WATER	DRY WEIGHT	ASH	WATER	DRY WEIGHT	ASH	STAGE
	mm.	*mgm.*		*mgm.*	*mgm.*	*mgm.*	*per cent*	*per cent*	*per cent*	
Mar. 27.....	2.21 ± .08	6.16	1.8							7–9 (Harrison)
Apr. 1.....	2.37 ± .11	7.32	1.8	4.98	2.34	.097	68.03	31.97	1.32	10–13
Apr. 6.....	2.75 ± .31	6.89	3.0	4.73	2.17	.070	68.58	31.42	1.01	15–20
Apr. 11.....	4.55 ± .99	8.43	11.2	5.70	2.73	.081	67.62	32.38	.96	26–31
Apr. 16.....	5.81 ± .14	6.98	28.1	4.51	2.48	.095	64.54	35.46	1.36	33±
Apr. 21.....	6.56 ± .15	7.85	35.9	5.53	2.33		70.38	29.62		36±
Apr. 26.....	9.45 ± .90	9.50	88.8	6.93	2.57		72.94	27.06		34–39
May 1.....	10.51 ± .77	9.78	118.6	7.54	2.24		77.08	22.92		39–41
May 6.....	11.09 ± .52	11.28	120.8	8.82	2.46		78.19	21.81		
May 11.....	11.76 ± .61	11.52	141.1	9.52	2.00	.20	82.63	17.37	1.74	42–43 Hatching
May 16.....	13.41 ± .61	25.3	95.2	22.87	2.43	.30	90.38	9.62	1.18	42–45
May 21.....	15.01 ± .75	27.34	123.6	25.89	2.45	.29	94.68	5.32	1.04	45 (yolk nearly gone)
May 26.....	16.75 ± 1.00	36.38	129.1	33.43	2.96	.38	91.86	8.14	1.04	46+ (eating?)
May 31.....	17.61 ± 1.33	63.64	85.8	58.66	4.98	.62	92.17	6.42	.97	
June 5.....	18.52 ± 1.64	58.42	108.7	53.47	4.95	.70	91.52	8.47	1.20	
June 10.....	18.65 ± 1.69	44.96	144.4	40.67	4.29	.45	90.45	9.54	1.00	
June 15.....	22.2 ± 2.33	83.56	130.8	75.68	7.88	.92	90.56	9.43	1.10	
June 20.....	25.18 ± 2.19	119.55	133.8			1.50			1.26	
June 25.....	24.7 ± 2.85	121.40	124.1	109.35	12.05	1.45	90.08	9.93	1.20	
June 30.....	29.93 ± 2.48	204.37	132.1	184.13	20.24	2.53	90.09	9.91	1.24	
July 5.....	29.8 ± 2.50	235.68	114.8	211.24	24.44	2.96	89.63	10.37	1.25	
July 10.....	32.0 ± 2.89	289.41	113.3	255.48	33.93	3.78	88.28	11.72	1.30	
July 15.....	37.21 ± 2.32	412.54	124.5	366.97	45.57	5.91	88.95	11.05	1.43	
July 20.....	39.78 ± 3.02	538.21	117.1	477.16	61.05	7.61	88.66	11.34	1.41	
July 25.....	41.94 ± 2.94	565.00	130.1	496.00	69.00	8.07	87.79	12.21	1.43	
July 30.....	45.10 ± 3.71	709.45	128.4	618.03	91.43	11.10	87.11	12.89	1.56	
Aug. 4.....	47.70 ± 4.17	798.68	135.8	694.13	104.55	11.55	86.91	13.09	1.45	
Aug. 9.....	48.50 ± 2.68	722.50	157.9	624.30	98.20	11.62	86.41	13.59	1.61	
Aug. 14.....	48.92 ± 2.36	785.63	148.8	673.77	111.86	13.30	85.76	14.24	1.69	
	50.50	784.50	164.1	667.25	117.25	15.25	85.05	14.95	1.94	Terrestrial
Aug. 19.....	48.70 ± 2.56	717.10	161.1	590.60	126.50	16.90	82.35	17.65	2.36	"

a straight line growth. Some deviation from this type of growth was found in the 13° data, more in the 19° data and still more deviation in the 27° data. It is very unlikely that the weight differs in these groups. High and low temperatures apparently affect the efficiency of the cardio-vascular mechanism so that atypical individuals are eventually produced. Under high temperature conditions the embryo folds around the yolk, develops rapidly and eventually straightens its axis; under the low temperature yolk is not readily utilized and the head and tail buds from the time of their formation extend away from the yolk mass rather than lie close to it.

In all the data provided by salamander collections under natural conditions, it may be noted that the "probable error of length" has a more or less constant ratio to the average length determinations of the various stages. The population may thus be considered to be fairly homogeneous concerning the individual growth rates.

Relation of Weight and Length.—Aside from the deviation between length and weight in the early embryonic stage, due to embryonic foldings, there are certain other fluctuations. Occasional samples from two other ponds compared with 1928 curves indicated that for a certain weight, there were considerable variations in length. In the period before the animals began to feed there was little difference in these values but later the differences were marked. When the 1929 curves are superimposed on the 1928 curves this relation is brought out clearly. The curves of linear growth and the weight curves practically coincide to the point *X* of Fig. 1. From this point to the period of metamorphosis the variation is great. A higher average "index of build" is found for the data of the first year as compared with that of 1929.

Time of Metamorphosis.—It seems quite probable that the actual time of metamorphosis under natural conditions is associated with the conditions of life in the pond. In August, 1929, the pond under consideration became dry. The growth weight as evidenced by the curve showed a marked slowing down toward the end of July, while in the previous year, under more favorable conditions, this was not evident until the first week in August. During this first year, in fact, there were specimens in the pond for at least two weeks after most of the salamanders had metamorphosed.[3] This laggard group was formed of

[3] R. G. Harrison (Correlation in the development and growth of the eye, etc. *Arch. f. Entw.-Mech.*, Bd. 120, 1929) has figured three curves for the post-embryonic linear growth of *A. maculatum* larvæ under laboratory conditions. These curves, which are sigmoid, indicate that the rate of growth is accelerated with increased feeding and, in contrast to the present data, that the length at metamorphosis is constant (47–50 mm.) under different feeding conditions. A similar curve is given by L. S. Stone (Heteroplastic transplantation of eyes between the larvæ of two species of *Amblystoma*. *Jour. Exp. Zoöl.*, Vol. 55, 1930).

relatively large specimens (Fig. 1, *a*, *a'*, *b*, *b'*). It seems quite probable that these specimens had not yet entered the third period of growth, *i.e.* the terminal period of slow growth. During the second year, when the pond became dry, two records, one before the pond became dry and the other immediately afterward, are available on the length and weight of recently metamorphosed specimens. In the first of these both values are higher than in the second. The second group was undoubtedly " forced " by the drying of the pond to metamorphose before reaching the stage at which the first group metamorphosed.

Allee (1911), who has studied the seasonal succession of pond fauna, has indicated that there is a periodic change in numbers of species and individuals found in forest ponds. There is an increase in numbers of species which is slow during the spring months and rapid in early summer, less marked in July and in late August the number falls to the spring value. There seems to be a correlation between the period of highest productivity of the pond as reported by Allee and the period of rapid growth of the salamanders recorded here.

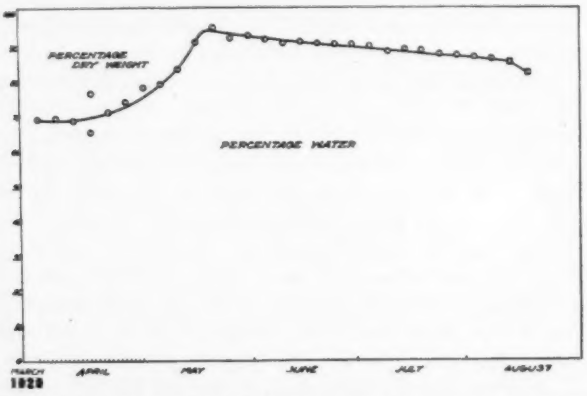

FIG. 3. Graph showing the relative percentage of water and organic substance in larval salamanders of different ages. Data of 1929.

Relations of Water, Solids and Ash to Growth.—The eggs shortly after they were laid had a weight of 7.32 mgm. consisting of 4.98 mgm. of water and 2.34 mgm. of solid, of which .097 mgm. was ash. During the embryonic period the dry weight was fairly constant. Actual increase was associated with increase in inorganic matter and water (Table I). The ash percentage, however, was practically constant while the water increased in this period from 68 to 94 per cent. After the animals began to eat, the dry weight increased considerably so

that the percentage of water decreased. To the period of metamorphosis there was a gradual increase of inorganic matter from 1 to 2 per cent. Water per cent decreased from 94 to 85 per cent and the percentage dry weight increased from 6 per cent to 15 per cent. This relationship is brought out in Fig. 3. Until the animals began to feed, growth was purely a process of hydration; afterwards it was due both to imbibition of water and to increase in organic and inorganic materials. These findings are in accord with the work of Davenport and Schaper on the Anura. Recently metamorphosed specimens showed still further decrease in the percentage of water content. Data on the percentage of water in older metamorphosed specimens (Table II) show that this early decrease may be later compensated. The proportion of dry weight, ash and water, however, seems to be variable for the land forms. Two specimens from an indoor aquarium in December showed a decrease in water content to 80 per cent body weight and an increase in inorganic matter to 4 per cent.

TABLE II

Showing the relative content of water, solids and ash in terrestrial stages of A. maculatum.

LENGTH	WET WEIGHT	$\frac{\text{LENGTH}^3}{\text{WEIGHT}}$	WATER	DRY WEIGHT	ASH	WATER	DRY WEIGHT	ASH
mm.	*grams*		*grams*	*grams*	*grams*	*per cent*	*per cent*	*per cent*
September 1929								
50.5	.603	214	.491	.112	.011	81.51	18.49	1.77
71	1.659	218	1.412	.247	.031	85.12	14.88	1.86
76	2.504	177	2.206	.298	.032	88.10	11.90	1.27
82	4.616	120	4.063	.553	.084	88.02	11.98	1.82
88	4.709	146	4.012	.697	.101	85.10	14.80	2.14
93	5.602	144	4.955	.647	.102	88.45	11.55	1.82
104	5.875	193	4.939	.936	.124	84.07	15.93	2.11
December 1928								
136	9.432	269	7.848	1.584	.427	83.21	16.79	4.53
138	8.792	301	7.044	1.748	.366	80.12	19.88	4.16

SUMMARY

1. Growth in weight of embryonic and larval *Ambystoma maculatum* from the time that eggs are deposited to the period of metamorphosis may be expressed as a single sigmoid curve.

2. The length curve, except for a short period before hatching when the embryonic axis is curved, is likewise sigmoid.

3. The *Ambystoma* population of a pond is quite homogeneous, the specimens metamorphosing at approximately the same time.

4. Under natural conditions the relation between weight and length from year to year seems to be constant during the stages before feeding. Later the relationships are variable because of feeding differences.

5. Growth to the time of food ingestion is associated with imbibition of water. Later growth to the time of emergence of the salamanders is correlated with a process in which the percentage of water content decreases. During this period the inorganic constituents gradually increase.

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THE EFFECTS OF TEMPERATURE CHANGES UPON THE CHROMATOPHORES OF CRUSTACEANS

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Temperature changes as they affected the chromatophores of crustaceans were not neglected in the researches of early investigators, those of Jourdain (1878) being the first recorded in the literature to consider the possible influence of this factor. At 5°–6° C., according to his observations, the rapidity at which color changes occurred in *Nica edulis* was appreciably reduced, ceasing entirely as the temperature approached nearer to zero. At this point the animals were almost transparent, except for areas partly covered with matted white spots. Jourdain removed the eyes of those crustaceans and noted that the reddish color assumed at room temperatures, under such circumstances, disappeared entirely when the temperature of the water was lowered only to reappear again on the restoration of the temperature to its former level. Matzdorff (1883) observed no effect whatever of either high or low temperatures upon the chromatophores of *Idotea tricuspidata.* Somewhat later however, Gamble and Keeble (1900), after a few observations upon *Hippolyte varians,* reported observable color response following exposure to heat and cold. Their specimens in common with most other crustaceans possessed several differently colored pigments, reds and yellows predominating, located with one exception in discrete bodies or chromatophores. During the day the reds and yellows were usually expanded, but at night these pigments were retracted into their chromatophore centers and if it were not for a blue pigment, diffused at this time throughout the tissues and free from any chromatophore. the animals would be colorless. Gamble and Keeble selected three of these transparent blue prawns, which they called "nocturnes," and placed one in water at 15.5° C. (60° F.), one in water at 8° C. and the last in water at 32° C. (93° F.). The first animal, in reality the control as it was kept under normal temperature conditions, turned greenish-brown as was to be expected. The second one at 8° C. maintained the nocturnal blue color, showing after thirty-five minutes some traces of recovery, though one hour later this was still incomplete.

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The prawn placed in 32° C. was almost immediately killed by the heat, but remained nevertheless a brilliant nocturne for several hours, even though during the first five minutes of this experiment the temperature descended to 28° C. (83° F.).

Menke (1911) experimenting with *Idotea,* produced a contraction of the chromatophores in about 15 minutes by raising the temperature of the water from 11.5° C. to 20.5° C. This contraction was sustained for about one hour when the pigment partially re-expanded. Five and one-half hours later on, lowering the temperature to 12° C., the chromatophores again became completely expanded. But if at this time, instead of lowering the temperature of the water, it was raised to 30° C., the chromatophores also re-expanded completely. Complete expansion was also produced by lowering the temperature from 14° C. to 4° C. Doflein (1910), working with *Leander xiphias* placed several specimens in complete darkness at 5°–8° C. for two to three days. At the lapse of this time the chromatophores and the tissues of the animal were completely impregnated with blue pigment, all other pigments being completely retracted into their respective centers. But as Fuchs (1914) points out, these results might follow either from continued exposure to cold or to darkness. Megušar (1911) working with *Gelasimus, Potamobius, Palæmonetes, and Palæmon,* observed an expansion of the chromatophores on the sudden transfer of any of these animals from water at 16°–18° C. to water at 25°–30° C. Similarly a contraction of the chromatophores followed a sudden transfer from water at 25°–30° C. to cooler water at 16°–18° C.

The results of these experiments are admittedly confusing, though as Fuchs (1914) observed, no reasonable doubt can be entertained as to the ability of temperature changes to produce an effect of some sort upon the pigmentary responses of the crustaceans. Further investigation of the subject was thought desirable in the hope of ascertaining, if possible, just how important a factor the action of heat and cold is in determining the distribution of the chromatophore pigment of this group. For this purpose a fresh water shrimp, kindly identified for me as *Macrobrachium acanthurus* Wiegmann by Dr. W. L. Schmitt of the United States National Museum, was selected as the subject of the experiments. These shrimps were obtainable in large numbers from the Arimao river and its immediate tributaries in the vicinity of the Harvard Biological Station, Cienfuegos, Cuba. I am happy to acknowledge my thanks and appreciation to Dr. Thomas Barbour for his assistance in putting the facilities of the Harvard Cuban Station at my disposal.

When caught, the chromatophore pigments of these shrimps were

more or less extended, giving the animal a reddish-brown color. This color varied somewhat with the size of the animal, the smallest being the lightest. As collected, the shrimps ranged from 2 cm. to 10 cm. in length, measured from rostrum to telson. Males varying from 2 cm. to 3 cm. in length were selected for the experiments. Females were rejected, as at this time their abdomens were practically opaque owing to the fact that they were carrying their eggs.

A word or two regarding the color changes of *Macrobrachium* will be an aid to the understanding of what is to follow. Taken to the laboratory and placed in white glazed porcelain bowls, the shrimps in daylight soon became transparent and colorless; microscopical examination of the abdomen and telson showing the chromatophores to be completely contracted. If such animals were placed upon a black background, they assumed a dark reddish color with the chromatophores well expanded. A somewhat superficial examination disclosed the presence of two types of chromatophore pigments, both apparently located in the same chromatophore, one being reddish-brown in color and the other yellow. These facts were derived from a microscopical examination of the living pigmentary units. Detailed histological study of the chromatophores was not attempted.

Occasionally under somewhat varying conditions, animals were seen with an unmistakable bluish color observed both in the light and in the dark. The blue pigment producing this color when examined under the microscope was clearly not confined to the chromatophores, but was free in the tissues, though its concentration did appear greater about the processes of the pigmentary centers. Gamble and Keeble (1900) reported that a blue color was the regular accompaniment of the nocturnal phase of *Hippolyte varians,* a phase characterized by the retraction of all other pigments into their respective centers. According to their statement, the blue pigment in *Hippolyte* responsible for the nocturnal coloration arises as a discharge product of the chromatophores, leaving these organs on the contraction of the yellow and red pigments, and apparently being derived from them. Left free in the tissues, the blue pigment is permanently divorced from its point of origin and persists in coloring the body of the prawns until it eventually disappears.

In these experiments upon *Macrobrachium acanthurus* determinations were first made of the action of heat and cold upon the color changes in normal shrimps. The method used was as follows: Two or three animals were placed in white porcelain bowls and covered with water at room temperatures. To this was added either warm or cold water, as desired, until the particular temperature demanded by the experiment was reached. Here it was either kept constant or

altered as necessary. The responses of the shrimps to temperature changes when kept upon a black background were tested in the same manner.

Numerous experiments with normal shrimps adapted to white backgrounds demonstrated conclusively that such animals darkened when exposed to temperatures either high enough or low enough to stimulate the chromatophores. Surprising as it may seem, once the response was complete, no criteria of any sort could be established separating the darkening produced by heat from that produced by cold. The color assumed in either circumstance was a deep red-brown, while microscopical examination showed the pigments of the chromatophores to be equally well extended at high and low temperatures. The protocols of the two following experiments may be taken as typical of many others:

2:13—28° C. Two colorless shrimps previously kept on a white background for a day were placed in a white porcelain bowl and small pieces of ice added to the water.
2:16—10° C. No change in color.
2:18—10° C. Shrimps appear slightly reddish.
2:22—15° C. Shrimps somewhat darker.
3:00—15° C. Shrimps a pronounced brown.
3:25—28° C. Shrimps still brown.
9:30—28° C. Shrimps colorless.

2:13—28° C. Two colorless shrimps previously kept on a white background for a day were placed in a white porcelain bowl and warm water gradually-added.
2:16—36° C. No change in color.
2:18—36° C. Shrimps faintly reddish.
2:21—36° C. Shrimps pronouncedly brown.
3:00—28° C. Shrimps colorless.

In all of the experiments the appearance of the red-brown color was more rapid at high temperatures than at low. With heat only 10–15 minutes were necessary to make the animal completely dark, while with cold 30–45 minutes were required. But regardless of whether the shrimps were exposed to heat or to cold, once the point of maximal darkening was reached, the intensity of the color was equal in both cases.

When the animals were subjected to warmth the lowest temperature capable of expanding the chromatophores was found to be 35° C., while temperatures as high as 40° C. could be withstood without subsequent death, though at this temperature and slightly below it, the shrimps remained motionless, and with the exception of gill movements showed no signs of life. Therefore, within the range of 35° C. to 40° C. the color of the shrimp is determined by the temperature of its en-

vironment rather than the type of background on which it happens to be. Similarly shrimps placed in water colder than 6° C. died immediately, while any temperature above 15° C. and, of course, below 35° C., failed to produce an expansion of the chromatophore pigment. Therefore, between 6° C. and 15° C. the color of the shrimp is also determined by temperature rather than background. It might be well to mention here that the temperature of the water in which the shrimps normally lived ranged from 25° C. to 30° C.

As a check upon these results and to determine whether there was any possibility of temperature changes producing a contraction of the chromatophore pigment, experiments similar to those just described were performed upon dark shrimps while they were upon a black background. But such animals when exposed to various temperatures ranging between 6° C. and 40° C. showed no alteration whatever in the expanded condition of their chromatophores.

Recovery of normal color and activity was the rule when shrimps subjected to effective temperatures were returned to water at about 28° C. But this recovery was more rapid in shrimps treated with warmth than those treated with cold. The former required but 30 to 40 minutes, and the latter 6 to 7 hours before normal temperatures and a white background again brought their chromatophore pigment to complete contraction.

Tests were also made of the responses of blinded shrimps to temperature changes, blinding being accomplished by cutting off the eyes at the base of the eye stalk. Shortly after this operation the pigment of the chromatophores began to expand and within an hour or so, regardless of background, this expansion was complete and the animals were red-brown in color. Shrimps in this condition placed in warm and cold water and left so for an appreciable length of time—two to three hours—showed no color change of any sort. Similarly shrimps anaesthetized with 0.05 per cent chloretone, failed to show color responses to either heat or cold. Neither high nor low temperatures are then capable of exerting any contracting effect upon the pigment of the chromatophores, even when these organs are removed from the influence of any stimuli directly or indirectly produced by the retina.

As Perkins (1928) has shown in *Palæmonetes,* the withdrawal of pigment into the centers of the crustacean chromatophore is controlled by a hormone elaborated in the eye stalks, a fact which was later substantiated by Koller (1928) on *Crangon* and *Leander.* Possibly then, as temperature changes acted to expand the chromatophore pigment, there was an inhibition by heat or cold of the mechanism controlling the production of this contracting secretion. Before such

an hypothesis could be tested, it was necessary to ascertain definitely whether or not such a secretion played a part in governing the chromatic responses of *Macrobrachium*. Consequently Perkins' experiments were repeated upon this animal. Five or six shrimps were paled by placing them upon a white background for a day or more, after which their eyes were removed and thoroughly macerated in 2 cc. of 0.7 per cent NaCl. One tenth cc. of the resulting solution was then injected into the abdomens of several shrimps in the dark condition and with well expanded chromatophores. In all cases the following reactions were noted: Shortly after injection, 5–10 minutes, the shrimps began to assume a bluish color which gradually increased in intensity until within 30 minutes it had reached its maximum; this was followed by a gradual retraction of the pigment into the chromatophore centers, a retraction which persisted until the shrimps had assumed a transparent blue color. These results closely parallel the effects reported by Perkins in *Palæmonetes,* even to the formation of the blue color, and offer complete substantiation of his findings. As control experiments 0.1 cc. of the extract was injected into the abdomens of several shrimps in the light condition with no observable effect. Similarly injection of 0.1 cc. of 0.7 per cent NaCl into blinded shrimps produced no pigmentary response.

The existence of a hormone produced by the action of light upon the retina and released into the circulation to affect a contraction of the chromatophore pigment is then demonstrated in the shrimps used in these experiments. Is the formation of this hormone in any way inhibited by either high or low temperatures? Apparently not, as the following experiments show. Two sets of extracts were prepared, one from the eyes of shrimps darkened on a white background by warm water (37° C.) and the other from the eyes of shrimps darkened on a white background by cold water (15° C.), both groups being subjected to their respective temperatures for the same length of time, namely 45 minutes. Two sets of blinded shrimps were then selected, one set being injected with 0.1 cc. of one extract and the other set with the same amount of the other extract. These animals were then replaced in water at room temperature and the results noted. In both cases these darkened shrimps paled within the specified length of time, but with this difference,—the blue color previously described appeared in only one out of three of the shrimps injected with the extract prepared from the eyes of animals kept at low temperatures, while it appeared in all of the shrimps injected with the extract prepared from the eyes of animals kept at high temperatures. Neglecting for the present the significance of this variation, it is obvious that extreme temperatures

in no way inhibit the manufacture or even the potency of the chromatophore-contracting hormone elaborated by the eye stalks.

This gives us a clue as to the manner in which heat and cold affect the chromatophores of crustaceans. Unfortunately, these experiments cannot give us a conclusive solution to this problem, though the data at hand strongly indicate a direct effect. Positive information is not to be derived from experiments on limbs or bits of integument isolated from the bodies of these shrimps, as the chromatophores of such excised pieces expand at once. Consequently subjecting such preparations to temperature variations accomplished no change in the distribution of their expanded chromatophore pigment. But since experiments on blinded and chloretonized shrimps give no evidence of any other type of response to temperature changes than those seen in normal light shrimps, and since neither heat nor cold affect the secretion of the chromatophore-contracting substances elaborated in the eye stalks, it seems reasonable to assume that the responses of the chromatophore pigment of crustaceans to high and low temperatures are direct.

A word or two in regard to the blue color and its relation to temperature changes. Keeble and Gamble (1903) state that the blue color observed in nocturnal *Hippolyte* disappears completely at 60° C., while, as shown in an earlier paper (Gamble and Keeble, 1900), this color is maintained at 8° C. under conditions that in other prawns kept at a somewhat higher temperature (15° C.) produce its loss. This latter observation is in accord with the experiments of Doflein (1910) on the occurrence of a blue color in *Leander* when the animals were kept for an extended period in darkness and cold. But aside from this, it is perhaps worthy of note that, as already mentioned, blinded animals injected with the extracts prepared from the eyes of shrimps subjected to cold showed only in one third of the cases a visible blue color, whereas blinded shrimps injected with an extract from the eyes of animals kept at high temperatures never failed to become pronouncedly blue. Furthermore, throughout the course of these experiments the blue color was repeatedly observed in connection with shrimps subjected to high temperatures, while the records disclose only one instance where it was seen in connection with shrimps exposed to low temperatures; a case where an animal kept at 6° C. for about 30 minutes turned blue when returned to 28° C. Perhaps this indicates a relationship between changes in temperature and the appearance of the blue color worthy of further investigation.

A survey of the work of previous investigators dealing with the action of heat and cold upon the crustacean chromatophore reveals a wide divergence of opinion. As we have already seen, Gamble and

Keeble (1900) claimed that both high and low temperatures produce or at least maintain a retraction of the pigment, a statement with which Jourdain (1878) and Doflein (1910) are in agreement as far as low temperatures are concerned. Menke (1911), on the other hand, reports that in *Idotea* extreme high and low temperatures tend to produce an expansion of the chromatophore pigment, though moderately high temperatures (20°–25° C.) lead to a contraction. Megušar (1911), however, observed an expansion of the chromatophore pigment with heat and a contraction with cold, though this author apparently did not subject his animal to temperatures lower than 15° C. In the most recent communication Koller (1927) maintains that temperature changes have no effect whatever upon the distribution of the chromatophore pigment in *Crangon*.

The results of the present writer's investigations are more in accord with those of Menke than with those of other workers, since Menke also observed an expansion of the pigment at both ends of the effective temperature scale. *Macrobrachium acanthurus* is a semi-tropical form, habituated to water normally remaining at 25°–30° C. the year around. Therefore, the response to temperature changes of such forms might reasonably be expected to vary somewhat from those seen by Menke in *Idotea*, a form adapted to life in cooler waters. Consequently we need not be greatly concerned when *Idotea* responds to temperatures of 20°–25° C. and Cuban shrimps do not. For the latter such temperatures are obviously not warm. The important feature is that for both types an expansion of the chromatophores is produced on exposure to temperatures either extremely high or extremely low.

Among the lizards and amphibians high temperatures as a rule produce a contraction of the chromatophores and low temperatures an expansion. Among the vertebrates in general, variations from this scheme are found in certain amphibians whose chromatophores are apparently insensitive to heat and among the fishes, where innervated melanophores react to warmth by expansion and to cold by contraction. The denervated melanophores of fishes respond, however, to temperature changes as do the chromatophores of lizards and amphibians. Since in these last two groups such reactions are presumably direct, and since they are certainly direct in denervated fish melanophores, it is permissible to say that among the vertebrates the independent response of the chromatophore to heat is a contraction and to cold an expansion. In the crustacean chromatophore where there is a high probability that reactions to temperature variations are direct, though this is admittedly not certain, an expansion of the chromatophore pigment is produced both by heat and cold. On the basis of our present

knowledge then there seems to be little resemblance between the pigmentary reactions to heat and cold in the vertebrates and the crustaceans.

Among the vertebrates, especially in the lacertilians, the ability of the pigment cells to respond to temperature changes is sometimes given a thermo-regulatory significance. But the crustacean chromatophore can certainly serve no such purpose, especially as the chromatic responses of this group are controlled by factors other than heat and cold. It is inconceivable, for instance, that the form used in these experiments would ever encounter in its usual environment temperatures high enough or low enough to bring about changes in the distribution of its chromatophore pigment other than the distribution determined by background or light intensity.

Summary

1. Expansion of the chromatophores of *Macrobrachium acanthurus*, a Cuban shrimp, follows immersion of these animals in fresh water at any temperature between 6° and 15° C. or between 35° and 40° C. This reaction occurs regardless of the background upon which the shrimp is placed. Between 15° C. and 35° C. the chromatophores of this shrimp expand when the animal is placed upon a black background and contract when the animal is placed upon a white background.

2. In blinded and chloretonized shrimps, the chromatophores are expanded and this expansion is in no way altered by changes in background or temperature.

3. Neither high nor low temperatures have any effect upon the potency or manufacture of the chromatophore-contracting substance elaborated by the eye stalks.

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